Award Number: DAMD17-99-1-9026

TITLE: Role of Nkx3.1 Homeodomain Protein in Prostate Carcinogenesis and Differentiation

PRINCIPAL INVESTIGATOR: Jeffrey D. Milbrandt, M.D., Ph.D.

CONTRACTING ORGANIZATION: Washington University
St. Louis, Missouri 63110

REPORT DATE: February 2000

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for public release; distribution unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.
Role of Nkx3.1 Homeodomain Protein in Prostate Carcinogenesis and Differentiation

Jeffrey D. Milbrandt, M.D., Ph.D.

Washington University
St. Louis, Missouri 63110

E-MAIL: JEFF@MILBRANDT.WUSTL.EDU

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

Prostate cancer is a significant health problem in aging American men. Approximately 350,000 American men were diagnosed and ~42,000 deaths were attributed to prostate cancer in 1998. Despite identification of chromosomal regions of instability that are associated with prostate cancer, there are an undetermined series of molecular events that lead to de-differentiation of prostatic epithelial cells and ultimately to prostate carcinoma. The specific genes responsible for neoplastic transformation of the prostate are largely unknown. Nkx3.1 is a homeodomain transcription factor that is expressed in an androgen-dependent and largely prostate specific manner. The Nkx3.1 gene localizes to the chromosomal region 8p21, a region that is often deleted in human prostate cancers. The goals of this study are to overexpress Nkx3.1 in vitro and determine the effects on growth/differentiation, patterns of target gene expression and tumorigenicity of prostate carcinoma cell lines. Expression patterns of Nkx3.1 in human prostate tumor specimens will be examined immunohistochemically, and microdissected prostate tumor samples will be used to search for Nkx3.1 mutations and/or evidence of hypermethylation. Specific cell types and androgen-dependent expression patterns of Nkx3.1 will also be monitored immunohistochemically in mouse prostates. Overall, our work will determine whether loss of Nkx3.1 function is a major cause of prostate cancer.
Opinions, interpretations, conclusions and recommendations are those of the author and are not necessarily endorsed by the U.S. Army.

Where copyrighted material is quoted, permission has been obtained to use such material.

Where material from documents designated for limited distribution is quoted, permission has been obtained to use the material.

Citations of commercial organizations and trade names in this report do not constitute an official Department of Army endorsement or approval of the products or services of these organizations.

X In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and use of Laboratory Animals of the Institute of Laboratory Resources, national Research Council (NIH Publication No. 86-23, Revised 1985).

N/A For the protection of human subjects, the investigator(s) adhered to policies of applicable Federal Law 45 CFR 46.

N/A In conducting research utilizing recombinant DNA technology, the investigator(s) adhered to current guidelines promulgated by the National Institutes of Health.

N/A In the conduct of research utilizing recombinant DNA, the investigator(s) adhered to the NIH Guidelines for Research Involving Recombinant DNA Molecules.

N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.
### TABLE OF CONTENTS

Role of Nkx3.2 Homeodomain Protein in Prostate Carcinogenesis and Differentiation

Page Numbers

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Front Cover</td>
<td>1</td>
</tr>
<tr>
<td>Standard Form (SF) 298</td>
<td>2</td>
</tr>
<tr>
<td>Foreword</td>
<td>3</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>4</td>
</tr>
<tr>
<td>Introduction and Body of Report</td>
<td>5-7</td>
</tr>
</tbody>
</table>
5. **Introduction.** The central purpose of this project is to ascertain the role of the Nkx3.1 homeodomain transcription factor in prostate cancer through its control of prostate cell differentiation and/or proliferation processes. The hypothesis is that Nkx3.1 is critical to normal prostate cell development and differentiation. Therefore, loss of Nkx3.1 expression leads to a de-differentiation of prostate epithelial cells and subsequently to development of prostate carcinoma.

6. **Body.** To determine the downstream target genes of Nkx3.1 in prostate cancer cells, we have developed a plan to create gain-of-function prostate cancer cells using adenoviral infection and gene profiling. To help develop and test this strategy we used an adenoviral delivery system to overexpress the zinc finger transcription factor, Egr1, in the human prostate carcinoma cell line LAPC4. The adenovirus expressing Egr1 construct included an EGFP tag that allows coexpression of EGFP and subsequent visualization of positive infected LAPC4 cells. Using fluorescent microscopy for EGFP visualization, we have optimized the conditions of adenoviral delivery to show >95% positively infected LAPC4 cells. RNA was purified from cells 24 hours after infection, reverse transcribed and gene expression was determined by quantitative RT/PCR analysis using a TaqMan. Through use of the TaqMan, relative levels of gene expression can be derived by measuring the cycle number at which the PCR product accumulation reaches a defined threshold (Ct). Relative expression of each gene was normalized against GAPDH and level of induction was determined after establishing a standard curve with serial dilutions of the cDNA sample containing the gene of interest. Our initial TaqMan survey of Egr1 induced genes related to previously reported Egr1 target genes including IGF-II, TGF-β, and PDGF-A. We determined that IGF-II was induced more than 500-fold in Egr1 infected LAPC4 cells compared to control virus infected LAPC4 cells. Furthermore, we showed a 50-fold induction of TGF-β and 12-fold induction of PDGF-A. As a further means to test for Egr1 target genes and their relative levels of expression in adenovirally overexpressing LAPC4 cells we employed the use of GeneChip analysis. The GeneChip arrays (Affymetrix) contain probe sets for approximately 5600 human genes. Using RNA from LAPC4 cells infected by control adenovirus versus Egr1 overexpressing adenovirus, we showed that the same Egr1 target genes were induced in LAPC4 cells. Therefore, using adenoviral infection of LAPC4 cells followed by TaqMan screening and GeneChip analysis provides an excellent method for us to identify the downstream targets of any transcription factor. Currently we have generated Nkx3.1 overexpressing adenovirus and this virus is being used to infect prostate cancer cell lines including LAPC4 and DU145 cells.

Nkx3.1 antisera have now been generated from rabbit antisera and were used to study immunohistochemical expression of Nkx3.1 in 20 human prostate tumor specimens. We found that Nkx3.1 expression was absent in the cancerous cells of approximately 60% of the human tumor samples. We hypothesized that loss of Nkx3.1 expression could be due to a mutation or changes in methylation. Initially, we have not identified mutations in the Nkx3.1 gene in five of the specimens analyzed. Loss of Nkx3.1 by changes in methylation is currently being investigated using methylation-specific PCR. To optimally perform methylation-specific PCR we have successfully employed the use of laser-capture microdissection to obtain pure prostate cancer cell DNA without
contamination of nearby normal prostate cell DNA. We have isolated and purified significant quantities of DNA and successfully amplified the Nkx3.1 gene from human prostate tumor specimens.

In addition to using the Nkx3.1 antisera on human prostate samples, we have used immunohistochemistry to examine expression of Nkx3.1 in the mouse prostate. We found that prostate expression of Nkx3.1 was localized to the luminal epithelial cells and was not detected in basal, neuroendocrine or stromal cells. To examine the androgen-dependent expression of Nkx3.1 in vivo, we examined the expression of Nkx3.1 after castration. We found that Nkx3.1 expression was progressively lost in the prostate within 6 days following castration. We next attempted to regain expression of Nkx3.1 by re-administering testosterone to castrated mice that were not immunohistochemically expressing Nkx3.1. Testosterone propionate implants (and BrdU) were given to mice for 3-4 days following 7 days of castration. We found that Nkx3.1 expression was restored within 4 days of testosterone replacement. Interestingly, proliferating cells (identified by BrdU immunohistochemistry) did not express Nkx3.1. Instead, Nkx3.1 staining was only detectable in non-proliferating prostate cells, consistent with our hypothesis that Nkx3.1 is important for prostate cell differentiation.

7. **Key Research Accomplishments.**

- Developed system for *in vitro* adenoviral overexpression of Nkx3.1. Will allow us to identify Nkx3.1 target genes and to investigate role of Nkx3.1 in differentiation/proliferation of prostate cancer cells.
- Identified through immunohistochemical study of 20 tumor specimens that Nkx3.1 was lost in approximately 60% of human prostate tumors.
- Isolated DNA and amplified Nkx3.1 by PCR after using laser-capture microdissection of cancerous cells of human prostate specimens.
- Determined that Nkx3.1 was expressed in the non-proliferating luminal epithelial cells of the prostate and not in surrounding basal, neuroendocrine or stromal cells.
- Nkx3.1 expression in the mouse prostate was lost after 7 days post castration, but Nkx3.1 expression could be regained after re-administering testosterone for 3-4 days.

8. **Reportable outcomes.** Rabbit anti-Nkx3.1 antisera

9. **Conclusions.** The observation that Nkx3.1 was lost in a majority of human prostate tumor specimens suggests that Nkx3.1 may indeed play a role as a tumor suppressor in prostate cancer. Our results suggest that the mechanism for loss of Nkx3.1 expression is not due to mutation of the Nkx3.1 gene, but appears to be a result of changes in methylation. Using the adenoviruses that we have now generated we will overexpress Nkx3.1 in human tumor cells and then profile the genes differentially expressed through GeneChip and TaqMan experiments. With the combined information of Nkx3.1 loss and downstream target genes we hope to provide important insight to elucidating the molecular genetic changes resulting in prostate cancer. Finally, using our
mouse castration and immunohistochemical studies we have identified the in vivo androgen-dependent and cell specific nature of Nkx3.1 gene expression. Indeed, we have shown that androgen withdrawal (castration) can extinguish Nkx3.1 expression and that expression is limited to the luminal epithelial cells of the prostate. Overall, we have provided evidence that shows the importance of Nkx3.1 as a tumor suppressor and will further potentiate our understanding of its role in prostate cancer by identifying Nkx3.1 target genes.

10. References. none

11. Appendices: none

Final Reports - Personnel receiving pay from the research effort:

Jeff Milbrandt, M.D., Ph.D.
John Svaren, Ph.D.
Toshi Araki, M.D., Ph.D.
Rakesh Nagarajan
Tim Fahrner