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PRINCIPAL INVESTIGATOR: Yuzhi Yin, Ph.D.
Edward Gelmann, M.D.

CONTRACTING ORGANIZATION: Georgetown University Medical Center
Washington, DC 20007

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13. ABSTRACT (Maximum 200 Words) NKX3.1 is a homeoprotein with prostate-specific expression in the adult mouse and human. We have identified the hexanucleotide DNA binding domain of NKX3.1. NKX3.1 gene targeting studies in mice have shown that the protein is a differentiating factor and suppressor of epithelial cell growth. We have described a genetic polymorphism, C154T, in humans that alters the amino acid sequence of the protein from arginine to cysteine at amino acid 52 (R52C). This polymorphism is present in 11% of the population. The variant protein has altered phosphorylation of serine 48, adjacent to R52C. The R52C variation results in a 70% decrease in in vitro phosphorylation of NKX3.1 by protein kinase C. Moreover, whereas DNA binding of wild type protein is regulated by phosphorylation, binding of NKX3.1 R52C to DNA is not affected by phosphorylation. The polymorphic site and an adjacent serine are critical for the activity of the C-terminal inhibitory region of NKX3.1 and may mediate binding of C- and N-terminal domains of the protein. Mutations of either R52 to C or S48 to A results in loss of C-terminal region inhibition of transcriptional activation by NKX3.1.				
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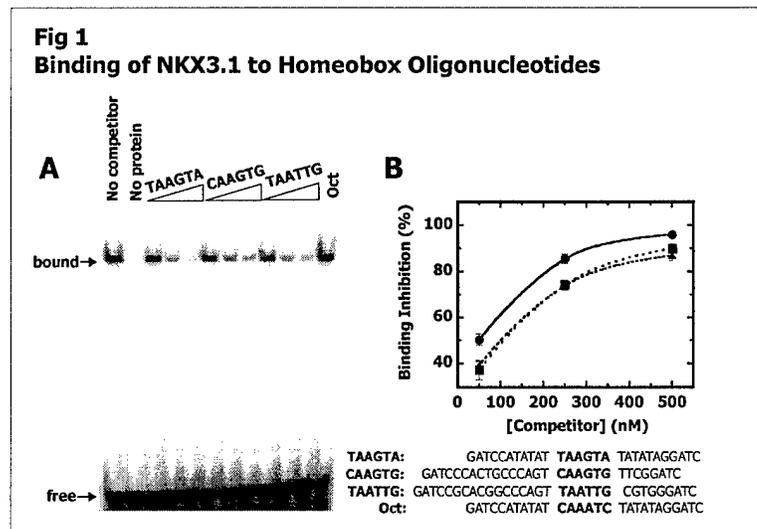
I. Introduction

This project included three tasks. Extensive progress has been made in this project and is summarized in this report. Each of the original tasks is listed below and progress in each is described. This grant was originally awarded to David Steadman who departed the University after 14 months. The work was assumed by Yuzhi Yin who completed the work on the project.

II. Identification of the NKX3.1 DNA Binding Domain

This task has been completed and the data published in Steadman, D.J., Giuffrida, D., and Gelmann, E.P. DNA binding sequence of the human prostate-specific homeodomain protein NKX3.1, *Nucleic Acids Research*, 28: 2389-2395, 2000. The complete set of figures and experimental details can be found in that paper.

Using a binding site selection assay with recombinant NKX3.1 protein we identified a TAAGTA consensus binding sequence that has not been reported for any other NK-class homeoprotein. By electromobility shift assay we demonstrated that NKX3.1 preferentially bound the TAAGTA sequence rather than the binding sites for Nkx2.1 (CAAGTG) or Msx1 (TAATTG). As shown in Figure 1 NKX3.1 specifically binds to the TAAGTA consensus binding site. Binding of MBP, WT NKX3.1 and R52C NKX3.1 to a radiolabeled probe containing either the NKX3.1 consensus sequence (TAAGTA) or an Oct-1 binding site (CTAAAC) was analyzed by gel mobility shift. Free probe and protein-bound complex are indicated with arrows. A 10-fold molar excess of unlabeled TAAGTA sequence was included as competitor, where indicated. Nucleotide sequences of the probes and competitor are listed at the bottom of the figure.



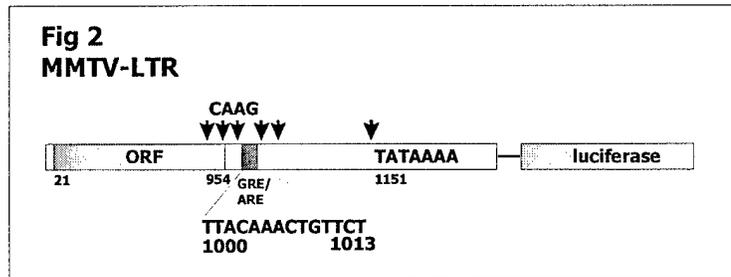
Free probe and protein-bound complex are indicated with arrows. A 10-fold molar excess of unlabeled TAAGTA sequence was included as competitor, where indicated. Nucleotide sequences of the probes and competitor are listed at the bottom of the figure.

The consensus binding site of a naturally occurring polymorphic NKX3.1 protein with arginine replaced by cysteine at position 52 was identical to the wild type binding sequence. The binding affinities of wild type and polymorphic NKX3.1 for the TAAGTA consensus site were very similar, with values of 2.3 and 2.6 nM, respectively. Wild type and polymorphic NKX3.1 specifically repressed transcription of luciferase from a reporter vector with 3 copies of the NKX3.1 binding site upstream from a thymidine kinase promoter. The data showed that among NK-family proteins NKX3.1 bound a novel DNA binding sequence and can behave as an *in vitro* transcriptional repressor.

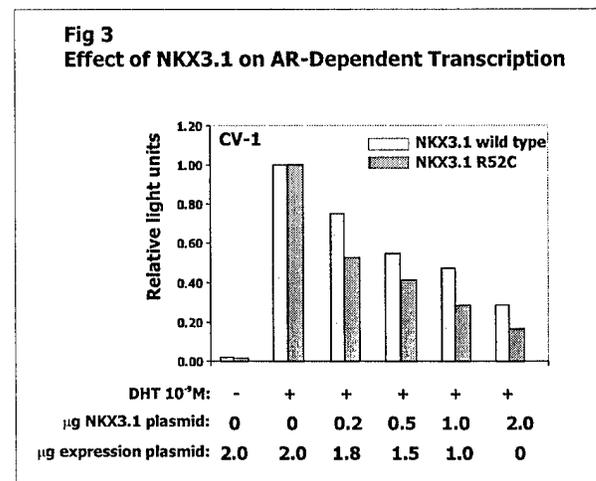
III. Analysis of the Effects of NKX3.1 on GRE-Induced Transcription

We had initially studied the interaction of NKX3.1 with an established promoter system,

MMTV-LTR. The MMTV-LTR has a known steroid hormone response element (SRE) that mediates transcriptional activation by both the glucocorticoid (GR) and androgen receptors (AR) (1-3). The SRE is located 449 nucleotides from the 3' end of the LTR and 151 nucleotides upstream from the TATA box. Six CAAG putative binding sites for NKX3.1 are in close proximity to the ARE (arrows in Figure 2). CV-1 cells were cotransfected with NKX3.1 expression vectors, an AR expression plasmid and a reporter plasmid containing the MMTV-LTR. Reporter activity was diminished up to 50% by the presence of NKX3.1 (Figure 3). The decreased level of transcription was not due to competitive binding of transcription factors by the increasing amounts of NKX3.1 expression plasmid added to the transfection. The cultures that received less than 2.0 μg of NKX3.1 expression plasmid DNA also received supplementary expression vector such that the total DNA content in each transfection was 2.0 μg . In the experiment shown in Figure 6 we saw more transcriptional suppression with NKX3.1 R52C than with wild type NKX3.1. This observation is consistent with the finding that NKX3.1 R52C binds more tightly to DNA than the wild type protein. Similar results were seen with glucocorticoid-induced transcription stimulated by dexamethasone (not shown).



It was the goal of Task 2 in the SOW to analyze the NKX3.1 binding sites in the MMTV-LTR. However two critical pieces of information arose from our experiments. First, reporter constructs were engineered with the left and right halves of the MMTV-LTR. We found, contrary to our hypothesis, that the left half of the LTR contained the region responsive to NKX3.1-mediated suppression and the right half with the ARE was unresponsive to NKX3.1. For this reason we abandoned the experiments proposed in the SOW because the left half of the MMTV-LTR had no sequences that resembled the consensus NKX3.1 binding site. We also found that NKX3.1 influenced transcription not only by binding directly to DNA, but also by interacting with other transcription factors. For example murine Nkx3.1 can potentiate transcriptional activation of smooth muscle γ -actin promoter by serum response factor (SRF) (4). Moreover Nkx3.1 can bind to SRF independent of DNA. We have shown that human NKX3.1 can also enhance SRF activation of smooth muscle γ -actin promoter. Since one of our near-term goals is to develop assays for different activities of the NKX3.1 protein, we examined the activity of different NKX3.1 constructs to potentiate transcriptional activation by SRF. Full-length wild-type NKX3.1 (NKX3.1 WT) potentiation of SRF can be seen in Figure 4A. Moreover, Figure 4B shows that SRF and NKX3.1 associate directly by coimmunoprecipitation. The related cardiac homeodomain protein NKX2.5 interacts with SRF via the homeodomain (5). NKX2.5 also interacts with other zinc-finger containing transcription factors such as GATA-4 via the homeodomain (6). In fact, binding of the N- and C-terminal regions of NKX2.5 inhibits both DNA and protein binding by the NKX2.5 homeodomain. Deletion of the C-terminal inhibitory region activates NKX2.5 binding to DNA and protein. By analogy, we hypothesize that the region including amino acids 48-52 of NKX3.1 is responsible for interaction with the C-terminus and thereby regulates NKX3.1 activity.



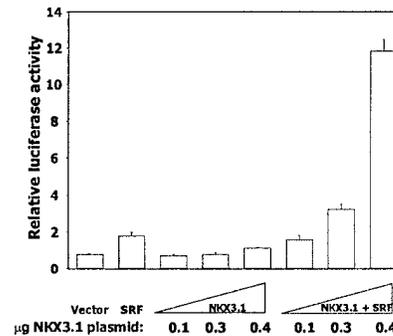
To gain a better understanding of the potential analogy between NKX2.5 and NKX3.1 and to determine the need for detailed molecular analysis of NKX3.1 we performed a Clustal alignment of NKX3.1 and NKX2.5. There is substantial homology in the homeodomain. T179 of NKX3.1 is not conserved in NKX2.5. In addition NKX3.1 S48 and R52 are not conserved in NKX2.5. Outside the homeodomain there is remarkably little conservation of amino acid sequence. In the C-terminal region there is conservation only of a short stretch that contains several tyrosines and prolines. This region may be a regulatory region that confers inhibitory activity to the C-termini of both proteins.

IV. Determine the Role of Amino Acid 52 in Phosphorylation of NKX3.1 by Protein Kinase C

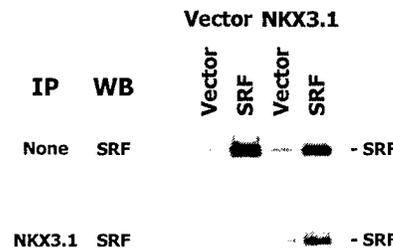
This task has been completed and the data are now included in the following paper: published in Gelmann, E.P., Steadman, D.J., Ma, J., Ahronovitz, A., Voeller, H.J., Swope, S., Abbaszadegan, M., Brown, K., Strand, K., Hayes, R.B., and Stampfer, M.J. Occurrence of *NKX3.1* C154T polymorphism in men with and without prostate cancer and studies of its effect on protein function. *Cancer Res*, 62:2654-9, 2002. In the course of analyzing tumor samples for *NKX3.1* mutations we found that some DNA samples contained a C→T polymorphism at nucleotide 154 (C154T) that coded for a variant NKX3.1 protein with a cysteine for arginine substitution at amino acid 52 (R52C) (7). The polymorphism lay N-terminal to the homeodomain in a region of the protein that is not conserved between mouse and human. Arginine 52 (R52) is four amino acids away from serine 48 (S48), the sole site for protein kinase C-mediated NKX3.1 phosphorylation *in vitro* and *in vivo*.

Despite being far upstream from the homeodomain that spans amino acids 124-183, phosphorylation of wild-type protein at serine 48 inhibits DNA binding. Alteration of the amino acid sequence by the polymorphism R52C or site-directed mutagenesis of the phosphorylation site to an alanine, S48A, abrogates phosphorylation at S48 by protein kinase C (Figure 2). Protein kinase C preferentially phosphorylates wild-type NKX3.1. In Figure 5A purified maltose binding protein or NKX3.1 fusion proteins (200 ng) were used as substrates in kinase reactions with protein kinase C (10 ng) and 0.11 μCi [γ - ^{32}P]ATP. Following the kinase reactions, samples were electrophoresed on a denaturing 10-20% gradient polyacrylamide gel. Phosphorylated proteins were identified by autoradiography (top). Western blotting using rabbit antiserum to maltose binding protein (5 μg) was used to control for protein loading (bottom). In Figure 5B peptides (30 μg) representing amino acids 43-54 of wild-type (P-WT), R52C (P-R52C) or S48A (P-S48A) NKX3.1 were used in an *in vitro* kinase assay with 10 ng protein kinase C and 0.11 μCi [γ - ^{32}P]ATP. Following the kinase reaction, samples were transferred to phosphocellulose discs, washed, and incorporated radioactivity was measured in counts per minute (CPM) by liquid scintillation counting. Amino acid sequences of the peptides are shown below the graph. In Figure 5C LNCaP cells were transfected with vectors expressing wild-type, R52C or S48A NKX3.1 with an N-terminal FLAG tag. The cells were treated with R1881 and 48 hr

Fig 4
A. NKX3.1 Activates SRF
Transcriptional Activity



B. Coimmunoprecipitation of NKX3.1 and SRF

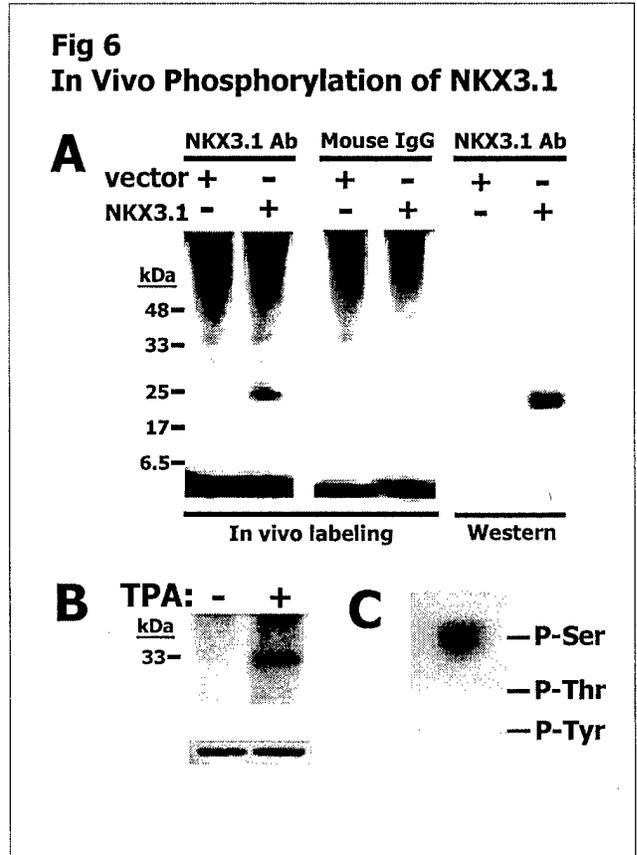
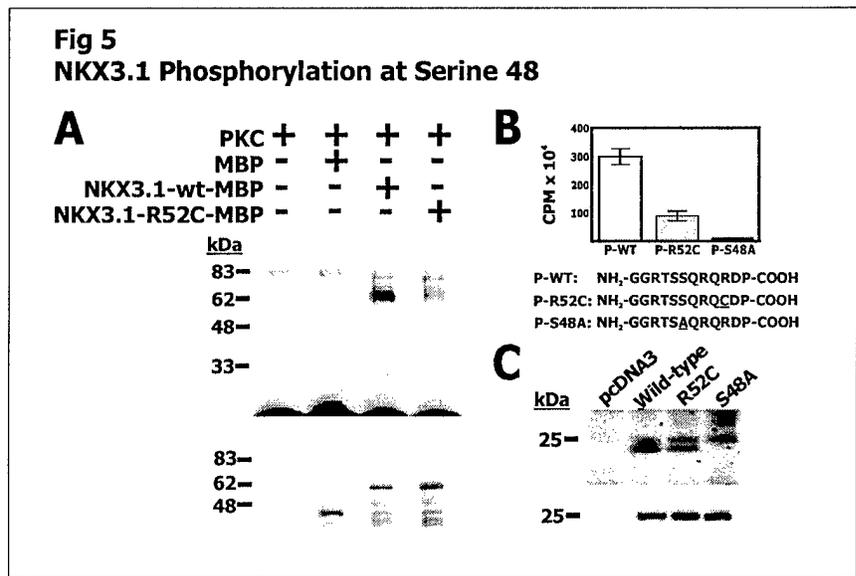


later the cells were exposed to 1 mCi/ml [³²P]orthophosphate. Cells were lysed and NKX3.1 was immunoprecipitated with an anti-FLAG antibody. Immunoprecipitants were electrophoresed and radiolabeled proteins were visualized by autoradiography (top). Western blotting using an anti-FLAG antibody (20 µg) was used to control for protein loading (bottom).

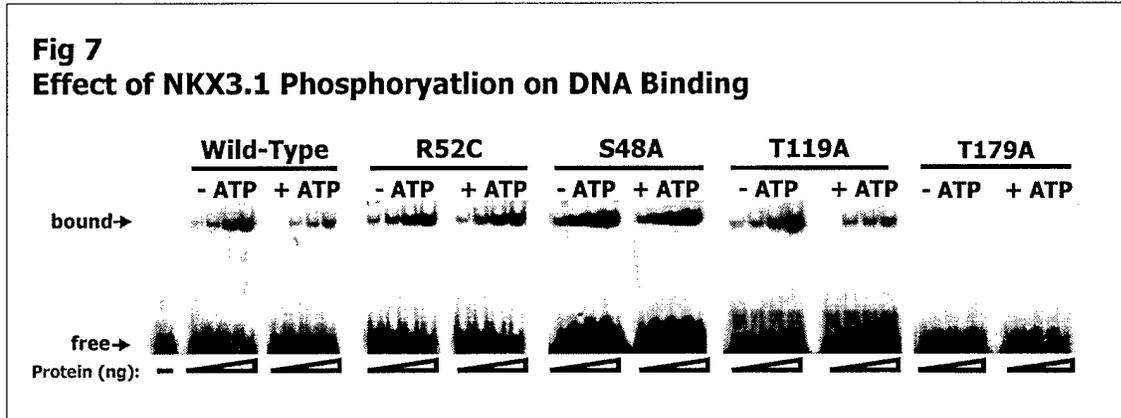
We also showed that endogenous NKX3.1 is phosphorylated in vivo after TSU-Pr1 cells are exposed to

TPA. In Figure 6A TSU-Pr1 cells were transfected with a wild-type NKX3.1 expression vector or empty vector. 48 hr post-transfection, cells were treated with 1 mCi/ml [³²P]orthophosphate. Cells were then lysed and NKX3.1 was immunoprecipitated with an anti-NKX3.1 antiserum or mouse IgG. Immunoprecipitants were electrophoresed by SDS-PAGE and phosphorylated protein was visualized by autoradiography. The same antibody used for immunoprecipitation of NKX3.1 was used in a western blot of lysates from cells transfected with either empty vector or NKX3.1 expression vector. In Figure 6B LNCaP cells were treated with R1881 and 48 hr later the cells were exposed to 1 mCi/ml [³²P]orthophosphate. Cells were then treated with or without TPA (100 nM) for an additional 30 min. Cells were lysed and NKX3.1 was

immunoprecipitated with an anti-NKX3.1 antibody, electrophoresed and radiolabeled proteins were visualized by autoradiography (top). Western blotting using an anti-NKX3.1 antibody (1.5 µg) was used to control for protein loading (bottom). In Figure 6C endogenous radiolabeled NKX3.1 was excised from a polyacrylamide gel, eluted and treated with 0.15 mg/ml trypsin. The digested protein was hydrolyzed with 6 N HCl for 1 hr at 105°C. Phosphoamino acids were separated by one-dimensional thin-layer electrophoresis. The identity of the phosphorylated amino acids was determined by autoradiography and comparison with phosphoamino acid standards. We also investigated the role of protein phosphorylation in regulating in vitro DNA binding as shown by gel retardation (Figure 7). R52C polymorphism affects phosphorylation-regulated DNA binding. Purified fusion proteins (200 ng) were treated with protein kinase C (10 ng) in the presence or absence of cold ATP. Following protein kinase C treatment, the proteins (2, 5, 10 or 25 ng) were used in gel shift assays with a radiolabeled



NKX3.1 consensus DNA binding sequence. Protein-bound DNA was separated from free probe by 8% native PAGE and the results were visualized by autoradiography. Note that addition of ATP to reactions with NKX3.1, labeled DNA and protein kinase C diminishes DNA binding of wild type NKX3.1 and of a control missense T119A mutant. But phosphorylation has little or no effect on DNA binding of NKX3.1 R52C or S48A. We also observed unexpectedly that the T179A mutant had decreased DNA binding by at least 100-fold.



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