Targeted Induction of Endogenous NKX3-1 by Small Activating RNA Inhibits Prostate Tumor Growth

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BACKGROUND. RNA activation (RNAa) is a small RNA-mediated gene regulation mechanism by which expression of a particular gene can be induced by targeting its promoter using small double-stranded RNA also known as small activating RNA (saRNA). We used saRNA as a molecular tool to examine NKX3-1’s role as a tumor suppressor and tested in vitro and in vivo antitumor effects of NKX3-1 induction by saRNA.

MATERIALS AND METHODS. NKX3-1 saRNA was transfected into human prostate cancer cells including LNCaP, CWR22R, PC-3, CWR22RV1, DuPro, LAPC4, and DU145. The transfected cells were used for analysis of gene expression by RT-PCR and immunoblotting, proliferation, apoptosis, and cell cycle distribution. PC-3 xenograft models were established in immunocompromised mice and treated with NKX3-1 saRNA.

RESULTS. NKX3-1 saRNA induced NKX3-1 expression in different prostate cancer cell lines, resulting in inhibited cell proliferation and survival, cell cycle arrest and apoptotic cell death. These effects were partly mediated by NKX3-1’s regulation of several downstream genes including the upregulation of p21 and p27, and the inhibition of VEGFC expression. Treatment of mouse xenograft prostate tumors with intratumoral delivery of NKX3-1 saRNA formulated in lipid nanoparticles significantly inhibited tumor growth and prolonged animal survival.

CONCLUSIONS. By revealing several important target genes of NKX3-1, our findings corroborated NKX3-1’s role as a tumor suppressor gene through direct regulation of the cell cycle and growth/survival pathways. This study also validated the therapeutic potential of saRNA for the treatment of prostate cancer via targeted activation of tumor suppressor genes.

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KEY WORDS: NKX3-1; prostate cancer; saRNA; RNAa; xenograft

INTRODUCTION

RNA activation (RNAa) is a small RNA-mediated gene activation mechanism [1–3]. Targeted activation of tumor suppressor genes via RNAa has been shown to elicit in vitro antigrowth or in vivo antitumor effects on different cancer cells including cancer of the bladder [4–7], liver [8–10], lung [11], breast [12], kidney [13], pancreas [14], lymphoma [15] and prostate [16,17], suggesting that RNAa may hold therapeutic potential for cancer.

NKX3-1 gene is regarded as a prostate specific tumor suppressor [18,19]. Deletion of Nkx3-1 in mouse causes phenotypic changes including prostatic epithelial...
lial hyperplasia, dysplasia [20,21], and prostatic intraepithelial neoplasia (PIN) [22]. In human prostate cancer, the chromosomal region 8p21 that harbors NKX3-1 gene [23] is frequently affected by loss of heterozygosity (LOH) [24,25] and expression of NKX3-1 is frequently lost or downregulated [25,26], while mutation of NKX3-1 is rarely found [27]. Restoring NKX3-1 function by vector-mediated ectopic expression can inhibit prostate cancer cell proliferation in vitro and tumor growth in vivo [19,28].

In the present study, we utilized RNAa as a molecular tool to dissect the molecular mechanism of NKX3-1’s tumor suppressor role and as a therapeutic to treat prostate cancer in a mouse xenograft model.

MATERIALS AND METHODS

saRNAs

saRNAs were designed and synthesized as previously described [1,3,29]. Two 21 nucleotides (nt) saRNAs targeting the NKX3-1 promoter at position −360 and −381 relative to the transcription start site (TSS) were designed (Supplementary Table I).

Plasmids

The plasmid that expresses NKX3-1 gene (pcDNA3.1-NKX3-1) was a gift from Dr. Gelmann of Columbia University as previously described [26,30].

Cell Culture and Transfection

Human prostate cancer cell lines were grown in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 2 mM l-glutamine, penicillin (100 U/ml), and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO₂ maintained at 37°C. The day before transfection, cells were plated in growth medium without antibiotics at a density of 50–60%. saRNA was transfected at a concentration of 50 nM using RNAiMax (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Plasmids pcDNA3.1 and pcDNA3.1-NKX3-1 were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for 72 hr. For stable overexpression, the transfected cells were selected with 1.5 mg/ml G418 for 3 weeks.

RNA Isolation and RT-PCR

Total cellular RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA). One microgram of RNA was used for cDNA synthesis using the ThermoScriptTM RT-PCR system (Invitrogen). The resulted cDNA was amplified by PCR using gene specific primers and primers for GAPDH as an internal control (Supplementary Table I).

Immunoblot Analysis

Immunoblot analysis was performed as previously described [31]. Briefly, lysates from transfected cells were clarified by centrifugation and supernatants were collected. Proteins were resolved on SDS-PAGE gels and transferred to nitrocellulose membranes. The resulted blots were blocked with primary antibodies specific for NKX3-1 (Santa Cruz Biotechnology), p21 (Cell Signaling), p27 (Cell Signaling), VEGF (Cell Signaling) and GAPDH (Cell Signaling). NKX3-1 protein levels in Figure 6B were detected using an anti-NKX3-1 antibody from Sigma (N6036). Blots were subsequently incubated with appropriate HRP-conjugated secondary antibodies and antigen–antibody complexes were visualized by chemiluminescence (Thermo Scientific).

Cell Proliferation Assay

Cell proliferation was assessed using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) by following the manufacturer’s instructions as previously described [31].

Cell Cycle Analysis

Cell cycle distributions were performed as previously described [31]. Briefly, transfected cells were fixed in 70% ethanol, stained in 1 ml of Krishna’s buffer for 1 hr at 4°C, and analyzed on a FACS Calibur flow cytometer (BD Bioscience, San Jose, CA). The resulted data was analyzed using The Modfit LT program (Verity Software House; Topsham, ME). Cell cycle distribution (G0/G1, S, and G2/M) was determined from only surviving cells with Sub-G0 cells excluded.

Apoptosis and Caspase 3/7 Activity Assays

Detection of apoptosis was performed using the Annexin V-PE Apoptosis Detection Kit II (BD Bioscience, San Jose, CA) according to manufacturer’s protocol. Caspase activity was measured by using the Caspase-Glo™ Assay (Promega, Madison, WI).

In Vivo Study

This study was approved by UCSF Institutional Animal Care and Use Committee (IACUC). Thirty athymic nude, homozygous male mice (nu/nu) at the
age of 4 weeks (Simonsen Laboratories, Gilroy, CA) were inoculated subcutaneously (s.c.) PC-3 cells (6.0 × 10^6) in 0.2 ml of PBS mixed with Matrigel (BD Biosciences) through a 27-gauge needle into the right lower flank of the mice. After 1 week when tumors reached an average weight of 0.29 ± 0.08 cm³, the tumor-bearing mice were randomly divided into three treatment groups receiving PBS, dsControl and dsNKX3-1-381 treatment respectively (Table I). All saRNAs were formulated in lipid nanoparticles (LNPs) by Alnylam Pharmaceuticals, Inc. (Boston, MA) as previously described [29]. The formulated saRNA was injected into tumor using a gauge-30 needle every 3 days for a total of 3 times. Mice body weight and tumor size were recorded every 3 days. Mice were removed from the study upon reaching predetermined endpoint criteria and recorded as a study death. The endpoint criteria were as follows: (1) the largest diameter of a tumor > 2.0 cm; (2) body condition score (BCS) of 2 or less; (3) weight loss of ≥20% from high body weight; and (4) active ulceration of tumor. At day 108 following the initial treatment, the study was terminated by sacrificing all surviving mice and tumors were removed and weighted. Tumor volume in cm³ was calculated by the formula: volume = (width)^2 × length/2.

### Statistical Analyses

The differences in continuous variables between treatments were assessed by Student t-test. The Mantel-Cox test was used to determine statistical significance of Kaplan-Meier survival curves. Significance was defined as P < 0.05.

### RESULTS

#### NKX3-1 saRNA Induced NKX3-1 Expression in Prostate Cancer Cells

By following rules derived from our previous studies [1,3], we designed two 21-nt saRNAs targeting the human NKX3-1 promoter at location -360 (dsNKX3-1-360) and -381 (dsNKX3-1-381) (Fig. 1A). We transfected these saRNAs into 7 prostate cancer cell lines irrespective of NKX3-1 status, including LNCaP, CWR22R, PC-3, CWR22RV1, DuPro, LAPC4 and DU145, and analyzed NKX3-1 mRNA expression 96 hr later by quantitative RT-PCR (RT-qPCR). As shown in Figure 1B, NKX3-1 mRNA was induced by dsNKX3-1-360 in LNCaP, PC-3 and LAPC4 cells with the induction level ranging from 1.5 to 2 fold. A consistent induction of NKX3-1 expression was observed in all seven cell lines following transfection with dsNKX3-1-381, with the level ranging from 1.5 (DU145) to 6.4 (LAPC4) fold. The target sites for dsNKX3-1-360 and -381 were only three basepair (bp) apart (Fig. 1A); it is unclear what determined their potency in RNAa activity. Previous studies have shown that RNAa is sensitive to target location [1,2]. Regardless, the observation that saRNAs targeting different locations induce the expression of the same target gene suggests that NKX3-1 activation is resulted from sequence specific targeting rather than off-target effects. In our subsequent experiments we focused on the more potent saRNA–dsNKX3-1-381. To further validate the RT-qPCR data, we transfected dsNKX3-1-381 into LNCaP, PC-3, CWR22R, CWR22RV1 and LAPC4 cells, and evaluated NKX3-1 protein expression by immunoblotting analysis. As shown in Figure 1C, NKX3-1 expression was consistently induced at the protein level in all cell lines, including PC-3 cells which do not have detectable NKX3-1 protein expression.

#### NKX3-1 saRNA Inhibited the Growth and Survival of Prostate Cancer Cells

Prostate cancer cells transfected with dsNKX3-1-381 exhibited a morphological phenotype of retarded growth and increased cell death compared to mock or control saRNA transfected cells (data not shown). As confirmed by colorimetric cell proliferation assays, dramatic reduction in viable cells was observed from day 2 to day 6 following transfection of dsNKX3-1-381 in all cell lines regardless of basal NKX3-1 expression levels (Fig. 2A). At day 6, dsNKX3-1-381 caused 66%, 58%, 56%, and 67% reduction of viable PC-3, LAPC4, CWR22R and CWR22RV1 cells respectively compared to mock transfection, while control saRNA transfection had no significant effect (Fig. 2A). To further evaluate how NKX3-1 activation may affect long-term cell

### Table I. Animal Groups and Treatment Regimens

<table>
<thead>
<tr>
<th>Treatment group</th>
<th>No. of mice</th>
<th>Treatment</th>
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</thead>
<tbody>
<tr>
<td>PBS</td>
<td>9</td>
<td>PBS: 150 μl, 3×</td>
</tr>
<tr>
<td>dsControl</td>
<td>10</td>
<td>dsControl-LNP (5 mg/kg), 150 μl, 3×</td>
</tr>
<tr>
<td>dsNKX3-1-381</td>
<td>9</td>
<td>dsNKX3-1-381-LNP (5 mg/kg), 150 μl, 3×</td>
</tr>
<tr>
<td>Total</td>
<td>28</td>
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survival, we performed colony formation assay on these cell lines. In LAPC4, CWR22R and CWR22RV1 cells, a single transfection of dsNKX3-1-381 completely abolished colony formation by these cells, while clonogenicity was significantly decreased in PC-3 cells following transfection with dsNKX3-1-381 (Fig. 2B). These results suggest that NKX3-1 activation by saRNA can inhibit cell proliferation and survival.

**NKX3-1 Activation Induced Apoptotic Cell Death and Arrested Cell Cycle Progression**

To determine the cellular mechanisms responsible for the antiproliferative effects of dsNKX3-1-381, we evaluated apoptotic cell death in cells transfected with dsNKX3-1-381 by flow cytometry following staining cells with Annexin V and 7-Aminoactinomycin D (7-AAD). As shown in Figure 3, dsNKX3-1-381 treatment caused a significant increase in both early and late apoptotic populations in all the cell lines tested compared to control treatments. To further confirm the apoptotic phenotype, we also assessed the activity of caspase 3/7, two key enzymes in the apoptotic signaling cascade, in cells treated with dsNKX3-1-381. We found that dsNKX3-1-381 treatment significantly increased caspase activity ($P < 0.001$) in all treated cells except CWR22R (Fig. 3C). We further determined cell cycle distribution in cells treated with dsNKX3-1-381.
by flow cytometry following propidium iodide (PI) staining of DNA. In all cell lines transfected with dsNKX3-1-381, we observed a significant increase in G0/G1 population and corresponding reduction in the S phase population compared to control treatments. In CWR22RV1 cells, an increase in the G2/M phase was also noted (Fig. 4A and B). This result suggests that dsNKX3-1-381 mainly arrests cell cycle at the G1 phase. Together, NKX3-1 saRNA has antigrowth effects via inducing apoptotic cell death and suppressing cell cycle progression.

Induction of p21 and p27, and Downregulation of VEGFC by NKX3-1 Activation

NKX3-1 is a transcription factor known to bind to the consensus sequence “TAAGTA” [30]. In a cDNA expression profiling study in PC-3 cells overexpressing NKX3-1, several cell cycle-related genes are shown to be regulated by NKX3-1 including p27 and p21 [32]. To explore the molecular mechanisms responsible for the inhibited cell proliferation and survival and increased apoptosis following NKX3-1 activation, we evaluated the expression of known NKX3-1 downstream genes including p21 and p27. As shown in Figure 5A, transfection of dsNKX3-1-381 into different prostate cancer cells caused significant induction of p21 and p27 protein expression. Similar results were obtained from cells that were transiently or stably transfected with a NKX3-1 expressing vector (Fig. 5B), thus validating the RNA interference results by confirming that the p21 and p27 induction is indeed through increased NKX3-1 expression. We also evaluated p21 and p27 mRNA expression in several cell lines after dsNKX3-1-381 transfection. p21 mRNA was induced by dsNKX3-1-381 in all cell lines but the saRNA had no apparent effect on p27 mRNA expression (Fig. 5C). In CWR22R cells stably expressing NKX3-1 (pcDNA3.1-NKX3-1), a 2.5 fold higher expression of p21 mRNA was detected compared to a stable line that carried an empty vector (pcDNA3.1), while p27 mRNA levels were not significantly changed (Fig. 5D). Taken together, RNA interference-mediated NKX3-1 activation modulates p21 expression at its mRNA and protein levels. Elevated NKX3-1 induces p27 mostly at the protein level, suggesting that the regulation is possibly through a post-transcriptional mechanism. Induction of p21 and p27 protein may contribute to the observed changes in cell cycle and apoptosis by NKX3-1 activation.

Previous studies have shown that NKX3-1 can repress VEGFC by directly binding to its promoter [32,33] and loss of NKX3-1 leads to increased
Fig. 3. Induction of apoptosis by dsNKKX3-1-381. Cells were transfected with 50 nM of the indicated saRNAs or mock transfected for 96 hr. A: Cells were stained with 7-AAD and a PE-conjugated antibody directed against Annexin V, and then analyzed by flow cytometry. Shown are examples of log fluorescence dot plots of Annexin V-fluorescein (x-axis) and 7-AADD (y-axis) stained cells. Viable cell population is in the lower left quadrant (double negative: Annexin V−/7-AADD−), early apoptotic cells are in the lower right quadrant (single positive: Annexin V+/7-AADD+), and late apoptotic cells are in the upper right quadrant (double positive: Annexin V+/7-AADD+). B: Flow cytometry data was plotted to compare apoptotic cell populations in each treatment (means ± SD from three independent experiments). Percentages of cells in early or late stage apoptosis are shown. C: Caspase 3/7 activity was determined by measuring luminescence of a cleavable reporter substrate. Data is plotted as the means ± SD from three independent experiments. *p < 0.05; **p < 0.01; ***p < 0.001 versus Mock.
VEGFC expression and lymphangiogenesis in late stages of advanced prostate cancer [33]. To support this view, we evaluated protein expression of VEGFC in prostate cancer cell lines transfected with dsNKX3-1-381. As shown in Figure 6A, downregulation of VEGFC protein expression was evident in LAPC-4, LNCaP, CWR22R, and PC-3 cells transfected with dsNKX3-1-381. No obvious change in VEGFC was observed in CWR22RV1 cells after transfecting dsNKX-3-1-381 (Fig. 6A). To further validate the regulation of VEGFC by NKX3-1 demonstrated by RNAa, we transfected pcDNA3.1-NKX3-1 into CWR22R cells and evaluated VEGFC protein expression. As shown in Figure 6B, ectopic expression of NKX3-1 dramatically increased NKX3-1 protein expression while significantly decreased VEGFC protein level compared to the empty vector control. These results thus confirmed VEGFC as an important downstream target of NKX3-1.

### In vivo Anti-Tumor Effect of NKX3-1 Activation Via RNAa

To test in vivo antitumor effects of NKX3-1 saRNA, we conducted a prostate cancer xenograft study with tumor burden and animal survival as the primary end points. After tumors were established \((0.29 \pm 0.08 \text{ cm}^3)\), saRNAs were administrated intratumorally every 3 days for 3 times (Fig. 7A). As shown in Figure 7B, dsNKX3-1-381 treatment caused a significant reduction in tumor burden compared to PBS or control saRNA treated tumors. At day 30 when all...
**Fig. 5.** Regulation of p21 and p27 expression by NKX3-1 overexpression. A: Cells were transfected with 50 nM of the indicated saRNAs or mock transfected for 96 hr. Protein levels for p21, p27 and GAPDH was detected by immunoblotting analysis. For CWR22R cells (bottom), samples for each treatment were in duplicates. B: pcDNA3.1 (pcDNA) or pcDNA3.1-NKX3-1 (pcDNA-NKX3-1) vector was transiently transfected into LAPC4 and CWR22R cells or stably transfected into CWR22R cells. Protein expression of NKX3-1, p21, p27 and GAPDH was detected 96 hr following transfection. C: Cells were transfected with 50 nM of the indicated saRNA or mock transfected. mRNA expression of p21 and p27 was assessed by RT-qPCR at 96 hr following transfection. Results are means ± SD of at two independent experiments. D: RNA was isolated from CWR22R stable pcDNA3.1-NKX3-1 cells and used for evaluating p21 and p27 mRNA expression by RT-qPCR (means ± SD, n = 2).

**Fig. 6.** Negative regulation of VEGFC by NKX3-1 overexpression. A: Cells were transfected with 50 nM of the indicated saRNAs for 96 hr. VEGFC protein levels were detected by immunoblotting assay. Protein levels of GAPDH were probed and used as a control for protein loading. B: pcDNA3.1 (pcDNA) or pcDNA3.1-NKX3-1 (pcDNA-NKX3-1) vector was transfected into CWR22R cells for 72 hr. Proteins were isolated from the transfected cells and analyzed for protein levels of NKX3-1, VEGFC and GAPDH by immunoblotting.
mice were still in the study, the average tumor weight for dsNKX3-1-381 group was 0.12 ± 0.2 cm³, an 85.1% (P < 0.0001) and 76.9% (P = 0.0098) reduction compared to that for PBS (0.81 ± 0.47 cm³) and dsControl (0.52 ± 0.21 cm³), respectively (Fig. 6B). At this time, three mice in the dsNKX3-1-381 treatment group were free of visible tumors. The average life span for PBS, dsControl and dsNKX3-1-381 group was 42.9 ± 1.6, 51.0 ± 5.5, and 74.4 ± 6.9 days, respectively, with a 73.4% (P = 0.006) and 45.9% (P = 0.016) increase in life span for dsNKX3-1-381 group compared to PBS and dsControl treatments respectively (Fig. 6C). Beginning from day 28, 6 (66.7%) mice treated with dsNKX3-1-381 were free of measurable tumors, of which, two remained tumor free till the end of the study and were considered as cured. Tumors in other four mice eventually relapsed and resulted in the death of these animals.

**DISCUSSION**

The fact that NKX3-1 is not affected by bi-allelic inactivation as other classic tumor suppressor genes [34] makes it a good candidate target for RNA-mediated activation to restore its function in the prostate for the purpose of inhibiting and reversing proliferative and metastatic phenotypes of prostate cancer. In this study, we identified a NKX3-1 specific saRNA which can activate NKX3-1 in different prostate cancer cell lines and suppress their growth by arresting the cell cycle and inducing apoptosis via at least partly modulating several downstream genes including p21, p27 and VEGFC. Furthermore, treating prostate cancer xenografts with NKX3-1 saRNA suppress tumor growth and extended animal survival.

Prostate cancer depends on androgen for growth and survival. Androgen deprivation therapy (ADT) remains the mainstay of treatment for advanced prostate cancer. Almost all patients eventually fail this treatment after initial response and develop castration-resistant prostate cancer (CRPC) [35,36]. Given the fact that NKX3-1 is an androgen regulated gene and plays an important tumor suppressor role by suppressing several oncogenic pathways, findings from this study may have implications in our understanding of CRPC development and also raise concerns for ADT. ADT, while primarily providing benefits to prostate cancer patients, may also lead to sequestration of tumor suppressors, such as NKX3-1, whose expression depends on AR signaling. It has been reported that androgen deprivation can cause unintended consequences including induction of epithelial–mesenchymal transition (EMT), which may ultimately lead to castration resistance [37].

Fig. 7. NKX3-1 saRNA decreased tumor burden and extended animal survival. A: Schematic representation of treatment regimens. B: Tumor growth curves measured before day 30 when all mice were in the study (means ± SD). C: Animal survival was evaluated by Kaplan–Meier analyses using the StatView statistical program. P values were determined by the Mantel–Cox test. D: Representative mouse pictures taken at day 28 showing subcutaneous tumor (dotted white circle line). Note the scar-like appearance resulted from the shrinkage of an established tumor in the right lower flank region (dotted white circle line) of a mouse from dsNKX3-1-381 treatment group and its enlarged view (far right image).
rationale for new strategies that minimize undesired effects of androgen ablation by activating NFKX3-1 as an adjuvant therapy to ADT.

Previous studies have observed that RNAa of a target gene can have functional consequences, such as the modulation of downstream gene expression, equivalent to those attainable by vector-based overexpression despite that RNAa can only offer an induction of expression in the range of a few-fold compared to much higher levels of overexpression achieved by vector systems [17,38]. This feature has been attributed to RNAa’s unique ability to augment productivity of an endogenous gene leading to a protein with more potent function than its exogenous counterpart. In consistency, RNAa of NFKX3-1 modulates several downstream genes to a similar extent as ectopically expressed NFKX3-1.

Our in vivo study successfully demonstrated the therapeutic benefits of NFKX3-1 saRNA delivered intratumorally. In all NFKX3-1 saRNA treated mice, their tumors were significantly shrunk, and many of them were free of measurable tumors 2–3 weeks following three dosings. However tumors in some mice treated with NFKX3-1 saRNA relapsed after initial response to the treatment and ultimately caused mortality. This was likely caused by residual tumor cells that had evaded the treatment and could otherwise be eliminated by additional dosings or systemic delivery in future studies.

CONCLUSIONS

Findings from the present study provides mechanistic insights toward NFKX3-1’s tumor suppressor role and reiterates NFKX3-1’s function in regulating multiple signaling pathways related to cell proliferation, cell cycle, apoptosis/survival, and invasion/metastasis. We also tested the therapeutic potential of NFKX3-1 saRNA and demonstrated its potent antitumor activity. Further study in clinically relevant animal models is warranted to evaluate the effectiveness of NFKX3-1 saRNA in treating advanced/metastatic prostate cancer.

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REFERENCES

NKX3-I saRNA Inhibits Prostate Tumor Growth


SUPPORTING INFORMATION

Additional supporting information may be found in the online version of this article at the publisher’s website.

Table SI. Sequences for dsRNAs and Oligonucleotide Primers