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·*Original Article*·

Effects of 9-cis retinoic acid on human homeobox gene *NKX3.1* expression in prostate cancer cell line LNCaP

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Abstract

Aim: To study the regulatory effects of 9-cis retinoic acid (RA) on the expression of human homeobox gene *NKX3.1* in prostate cancer cell line LNCaP. **Methods:** Flow cytometry, reverse transcriptase polymerase chain reaction and Western blotting were performed to evaluate the effects of 9-cis RA on *NKX3.1* expression and cell cycle of LNCaP cells. To identify a regulatory region within the *NKX3.1* promoter contributing to the regulation induced by 9-cis RA, we have constructed an *NKX3.1* promoter-reporter plasmid, pGL_3-1040bp, and its 5'-deletion mutants, which were transfected into LNCaP cells with treatment of 9-cis RA in indicated concentrations. **Results:** With the treatment of 9-cis RA, the *NKX3.1* promoter activity was increased in reporter gene assay and *NKX3.1* expression was enhanced at both mRNA and protein levels in LNCaP cells. We found that the region between –936 and –921 in the upstream of *NKX3.1* gene involved the inducible regulation by 9-cis RA treatment. In flow cytometry, 9-cis RA treatment caused accumulation of cells in the G_1 phase of the cell cycle and a fewer cells pass through to G_2/M. **Conclusion:** Our results demonstrated that 9-cis RA as a differentiating agent can arrest prostate cancer cells in G_1 phase and reduce cell mitosis, and upregulate the expression of human homeobox gene *NKX3.1*, which is thought to play an important role in prostate differentiation and to act as a tumor suppressor gene in the prostate. *(Asian J Androl 2006 Jul; 8: 435–441)*

Keywords: *NKX3.1* gene; 9-cis retinoic acid; gene expression; prostate cancer cell

1 Introduction

Human homeobox gene *NKX3.1* plays a critical role in the regulation of growth and differentiation of the prostate. It exhibits prostate-specific expression [1]. Loss of *NKX3.1* expression has been implicated in prostate development [2], tumorigenesis [3] and progression of prostate cancer [4]. *NKX3.1* maps to chromosome band 8p21 [5], which is a region frequently lost in prostate cancer [6, 7]. In mice, targeted disruption of *Nkx3.1* leads to prostatic epithelial hyperplasia and dysplasia [8], and over-expression of exogenous *NKX3.1* suppresses growth and tumorigenicity in human prostate carcinoma cell lines [3].

9-cis retinoic acid (RA) is a natural metabolite of retinoic acid in which the all-*trans* configuration of the polyene side chain is replaced with a cis configuration at the 9 position. 9-cis RA has demonstrated antiproliferative and/or differentiating activity in *in vitro* models of prostate cancer [9], breast cancer [10, 11], leukemia and lym-

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phoma [11, 12], lung cancer [13], and head and neck cancer [14]. *In vivo*, 9-cis RA has significant anticarcinogenic activity in the rat mammary gland [15, 16] and in the rat colon [17]. 9-cis RA is thought to be a differentiating agent and an inhibitor of carcinogenesis.

To demonstrate the relationship between 9-cis RA and *NKX3.1* in prostate differentiation and cancer, we investigated the regulation of *NKX3.1* gene expression by 9-cis RA treatment in the prostate cancer cell line LNCaP.

2 Materials and methods

2.1 Cell culture and treatment

The human prostate cancer cell line LNCaP obtained from the American Type Culture Collection was grown at 37ºC in 5% CO_2 with RPMI 1640 media (GIBCO BRL Grand Island, NK, USA) supplemented with 10% fetal bovine serum (GIBCO BRL Grand Island, NK, USA) and ampicillin 100 U/mL and streptomycin 100 U/mL.

9-cis RA stocks were prepared in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO, USA) at a concentration 1 000-fold higher than the working concentration. Fresh culture media were premixed with the stock and then added to triplicate bottles. Media and 9-cis RA were replenished every day. Controls received DMSO vehicle at a concentration equal to that in 9-cis RA treated cells.

2.2 Reverse transcriptase polymerase chain reaction (RT-PCR)

Total RNA was extracted from LNCaP cells with TRIzol reagent (MBI Fermentas, Hanover, MD, USA) following the manufacturer's instructions after 48 h of treatment with 9-cis RA. The expression of *NKX3.1* mRNA was determined by reverse transcriptase polymerase chain reaction (RT-PCR) with the M-Mul V reverse transcriptase in the presence of random hexamer primer. PCR primers for *NKX3.1* were as follows: 5'-GTACCTGTCGGCCCCTGAACG-3' (sense) and 5'-GGACCAGAGGCACATAATGTCG-3' (antisense); for *β-actin* were 5'GTGGG GCGCCCAGGCACCAC-3' (sense) and 5'-CTCCTTAATGTCACGCACGATTT-3' (antisense). PCR conditions were denaturation at 94ºC for 2 min, followed by 28 cycles at 94ºC for 30 s, 63ºC for 30 s, and 72ºC for 40 s, followed by heating at 72ºC for 8 min. A total of 550 bp of *β-actin* mRNA was amplified and used to normalize the quantity of the *NKX3.1* mRNA in RT-PCR.

2.3 Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

After 48 h of treatment with 9-cis RA, LNCaP cells were harvested and lysed with cell lyses buffer (50 mmol/L Tris-HCl [pH 8.0], 150 mmol/L NaCl, 0.1% sodium dodecyl sulphate [SDS], 100 μg/mL PMSF, 1 μg/mL aprotinin and 1% NP-40). Cell extracts were quantified by using the bicinchoninic acid (BCA) method. For Western blot analysis, 40 μg of cell extracts were separated on 10% SDS-PAGE and then transferred to nitrocellulose membrane. After being blocked and washed, the membrane was incubated with human specific anti-*NKX3.1* antibody (RDI, Concord, MA, USA) at 4ºC for 12 h, followed by incubation with peroxidase-labeled second antibody for 1 h at room temperature, and immunoreactive bands were visualized by enhanced chemiluminescence (ECL, Santa Cruz, CA, USA). *β-actin* (Sigma, St. Louis, MO, USA) was used to normalize the quantity of the protein on the blot.

2.4 Construction of luciferase reporter plasmids

pGL_3-1040bp *NKX3.1* promoter was constructed as previously described [18] and its 5'-deletion mutants were generated by PCR method using pGL_3-1040 as the template. The primers used in PCR were one lower primer, PF+8, and eight upper primers: PD-945, PD-936, PD-920, PD-904, PD-883, PD-869, PD-591 and PD-391 (sequences shown in Table 1). The PCR was conducted at 94ºC for 2 min followed by 35 cycles at 94ºC for 30 s, 60ºC for 30 s, and 72ºC for 1 min. The PCR products were separated by 1.0% agarose gel electrophoresis and purified with QIA quick gel extraction kit, then cut with *Xho* I and *Sac* I and inserted into the equivalent site of pGL_3-basic vector (Promaga, Madison, WI, USA) to generate eight constructs that were designated pGL_3-953, pGL_3-944, pGL_3-928, pGL_3-912, pGL_3-891, pGL_3-877, pGL_3-599 and pGL_3-399. All of them were confirmed by restriction enzyme digestion and DNA sequencing.

A 16 bp cis-element from –936 to –921, called 9-cis RA inducible element (RAie), involving the 9-cis RA inducible regulation on *NKX3.1* promoter has been identified by 5'-deletion mutation analysis above. To confirm its response to 9-cis RA treatment, the 16 bp RAie sequence (sense 5'-TCGAGCTTCTTTTTTTTACGGGAG CT-3' and antisense 5'-CCCGTAAAAAAAAGAAGC-3') was synthesized *in vitro* and inserted into the upstream of an SV40 promoter-luciferase reporter in pGL_3-pro-

Table 1. The primers used in mutagenesis analysis.

Names	Sequences of primes
PF+8	5'-GGCCTCGAGCGCACCGCTTTC ACTTTC-3'
PD–945	5'-CGCGAGCTCAGACCCTGTCCG TAAAAA-3'
PD–936	5'-CGCGAGCTCCCGTAAAAAAAA GAAGAG-3'
PD–920	5'-CGCGAGCTCAGAAAAGACAAG GAAAGA-3'
PD–904	5'-CGCGAGCTCGAAAATACTTCC ATAATC-3'
PD–883	5'-CGCGAGCTCGTTCCACTTTCG TCTGTT-3'
PD–869	5'-CGCGAGCTCCTGTTGTCACGG TACCGT-3'
PD–591	5'-CGCGAGCTCACCACTTCTGCA ACGGGA-3'
PD–391	5'-CGCGAGCTCAATTGGCTCTGA CGGTCC-3'

moter vector (Promaga, Madison, WI, USA) at *Sac* I and *Xho* I sites to generate the pGL_3-RAie-promoter plasmid. The construct was confirmed by DNA sequencing analysis.

2.5 Transient transfection and 9-cis RA treatment

For luciferase reporter assay, LNCaP cells were seeded in 24-well plates and transfected with lipofectimine 2000 after 48 h of passage. Each well included approximately 1.5×10^5 cells, 1.0 μg pGL_3 constructs, 0.04 μg internal control plasmid pRL-TK, 2 μL lipofectimine 2000 and 500 μL RPMI 1640 media without serum and antibiotics. The media was changed to RPMI 1640 with 10% fetal bovine serum 6 h later. The transfected cells were treated with 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} mol/L of 9-cis RA, respectively, for 48 h, then the cells were harvested for dual-luciferase assay.

2.6 Dual-luciferase reporter assay

The activities of firefly luciferase in pGL_3 and Renilla luciferase in pRL-TK were determined following the dual-luciferase reporter assay protocol recommended by Promega (Madison, WI, USA). The cells were rinsed twice with phosphate-buffered saline (PBS) and cell lysates were prepared by manually scraping the cells from culture plates in the presence of 1 × passive lysis buffer. (Dual-Luciferase Assay System Kit, Promega, Madison, WI, USA). Twenty microliters of cell lysate was transferred into the luminometer tube containing 100 μL luciferase assay reagent (LAR), and firefly luciferase activity (M_1) was firstly measured then Renilla luciferase activity (M_2) was measured after adding 100 μL of Stop & Glo Reagent. The results were calculated and expressed as the ratio of M_1/M_2. The experiments were carried out three times with double replicates. The data are presented as mean ± SD.

2.7 Cell cycle experiments

LNCaP cells were plated in 50 mL bottles and treated with the 10^{-6} mol/L of 9-cis RA or vehicle (0.1% DMSO) for 72 h. The cells were scraped into medium, washed twice with PBS and fixed with ice-cold 70% ethanol overnight at 4ºC. After brief centrifugation, cells were washed once with PBS and incubated for 30 min at 37ºC in PBS containing 40 μg/mL of propidium iodide and 100 μg/mL of DNase-free RNase. Flow cytometric analysis was performed to detect the cell cycle and apoptosis. Sub-G_1 peaks were calculated and presented the apoptosis of the cell.

2.8 Statistics

Dates are presented as the mean ± SD. Comparisons between groups were done using paired *t*-test. $P < 0.05$ is considered significant.

3 Results

3.1 Activation of NKX3.1 promoter activity in LNCaP cells by 9-cis RA treatment

To observe the effects of 9-cis RA on *NKX*3.1 promoter activity, LNCaP cells were seeded in 24-well plates and transfected with pGL_3-1040 by using lipofectimine 2000. After the transfection, the cells were treated with 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8} mol/L of 9-cis RA, respectively, for 48 h, and then cells were harvested for dual-luciferase assay. The results in Figure 1 show that 9-cis RA enhanced 1040bp *NKX3.1* promoter activity in a dose-dependent manner.

3.2 Identification of a region in NKX3.1 promoter contributing to the 9-cis retinoic acid inducible regulation

To identify a regulatory region within the *NKX3.1* promoter contributing to the regulation induced by 9-cis

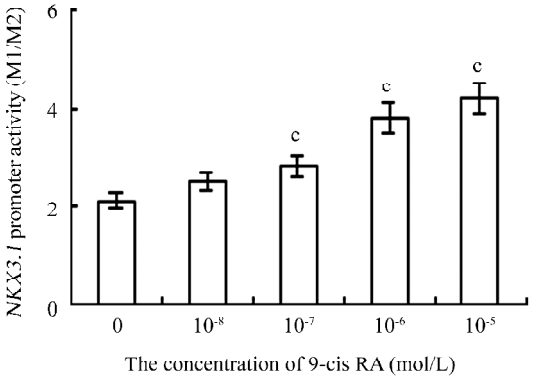

Figure 1. Effect of 9-cis retinoic acid (RA) on *NKX3.1* promoter-luciferase reporter activity. *NKX3.1* promoter activity was represented by the ratio of firefly luciferase activity (M_1) in pGL_3 and Renilla luciferase activity (M_2) in pRL-TK. The results were presented as mean ± SD from 4 wells. $^cP<0.01$, compared with the control (without the treatment of 9-cis RA).

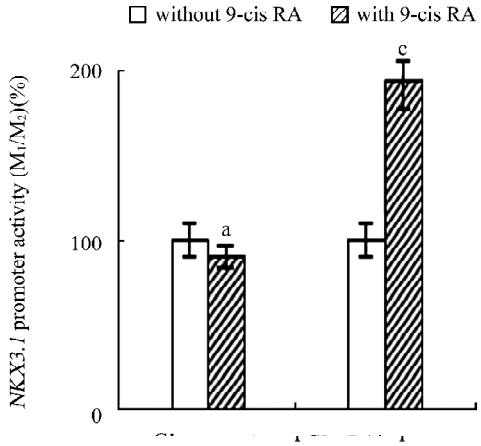

Figure 3. RAie mediated stimulation in reporter activity. *NKX3.1* promoter activity was expressed as relative luciferase activities (M_1/M_2). The data were represented as mean ± SD ($n = 6$). $^aP > 0.05$, $^cP < 0.01$, treatment with 9-cis RA (stripe bars) *vs.* without 9-cis RA (hollow bars) in pGL_3-RAie-promoter transfection.

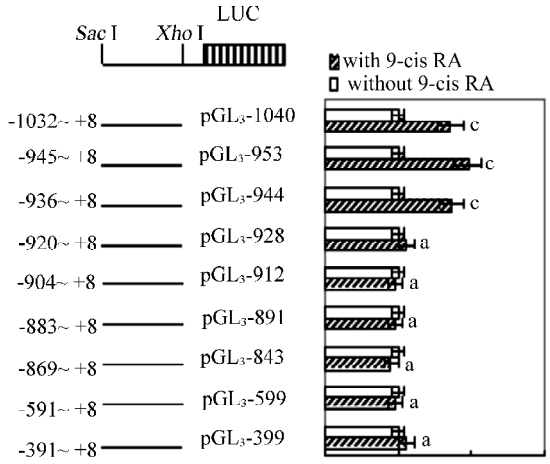

Figure 2. *NKX3.1* promoter 5'-deletion constructs and the promoter activity assay in LNCaP cells with 9-cis retinoic acid (RA) treatments. *NKX3.1* promoter activity was expressed as relative luciferase activities (M_1/M_2) that are the ratio of firefly luciferase activity (M_1) in pGL_3 plasmid and Renilla luciferase activity (M_2) in pRL-TK plasmid. The data were represented as the mean of six individual values ± SD ($n = 6$). $^aP > 0.05$, $^cP < 0.01$, treatment with 9-cis RA (stripe bars) *vs.* without 9-cis RA (hollow bars).

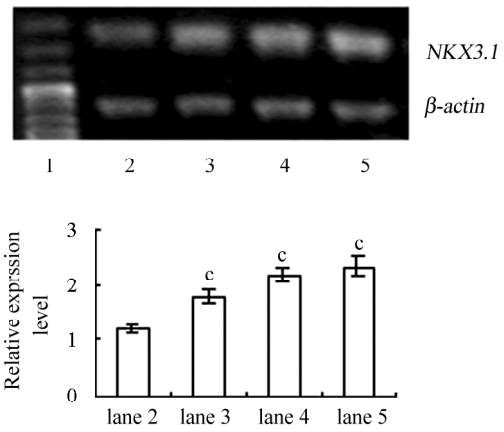

Figure 4. Effect of the 9-cis retinoic acid (RA) treatment on *NKX3.1* mRNA expression in dose-dependent manner. The LNCaP cells were cultured in 25 mL bottles for 48 h after treatments with 10^{-5}, 10^{-6} and 10^{-7} mol/L of 9-cis RA. 1, DNA marker (their molecular sizes are 200, 300, 400, 500 and 600 bp from top to bottom); 2, control without the treatment of 9-cis RA; 3, with 10^{-7} mol/L of 9-cis RA treatment; 4, with 10^{-6} mol/L of 9-cis RA treatment; 5, with 10^{-5} mol/L of 9-cis RA reatment. The relative expression levels are presented as the ratio of densities of *NKX3.1* and *β-actin* gene cDNA bands. The results are expressed as mean ± SD ($n = 3$). $^cP < 0.01$, the lanes 3, 4, 5 *vs.* lane 2 (control).

RA treatment, we have constructed pGL_3-1040bp *NKX*3.1 promoter and its 5'-deletion mutants, pGL_3-953, pGL_3-944, pGL_3-928, pGL_3-912, pGL_3-891, pGL_3-877, pGL_3-599 and pGL_3-399, which were transfected into LNCaP cells treated with 10^{-6} mol/L of 9-cis RA. The results shown in Figure 2 indicate that a

region between –936 and –921 in the upstream of *NKX3.1* gene was involved in the 9-cis RA inducible regulation. This 16 bp sequence was named as 9-cis RA inducible element (RAie).

3.3 The functional assay of RA inducible element mediating 9-cis RAie

To examine the potential role of RAie on mediating 9-cis RA inducible regulation, we inserted the double strands of RAie sequence into the upstream of SV40 promoter-luciferase reporter in pGL_3-promoter plasmid (Promega, USA) to generate the pGL_3-RAie-promoter, then the construct was used in a transient transfection experiment to observe the effects of RAie on mediating 9-cis RA inducible upregulation of the luciferase reporter activity. The RAie mediated stimulation is seen in reporter activity in Figure 3. The data suggested that RAie was a functional *cis*-element contributing to 9-cis RA inducible regulation.

3.4 Upregulation of NKX3.1 mRNA expression induced by 9-cis RA treatment

RT-PCR was performed to investigate the effects of 9-cis RA on the *NKX3.1* mRNA expression. LNCaP cells were cultured in 25 mL bottles and treated with 10^{-5}, 10^{-6} and 10^{-7} mol/L of 9-cis RA for 48 h. As shown in Figure 4, the expression of *NKX3.1* mRNA was enhanced by 9-cis RA treatment in a dose-dependent manner (lanes 3–5, in Figure 4).

3.5 Upregulation of NKX3.1 protein expression induced by 9-cis RA treatment

Western blot analysis was carried out to detect the effect of 9-cis RA on NKX3.1 protein expression in LNCaP cells that were treated with 10^{-5}, 10^{-6} and10^{-7} mol/L of 9-cis RA for 48 h. As expected, NKX3.1 protein expression was significantly upregulated by 9-cis RA treatment in a dose-dependent manner (lanes 2–4 in Figure 5).

3.6 Effects of 9-cis RA treatment on LNCaP cell cycle

To determine whether 9-cis RA treatments change the progression of cells through the cell cycle, cells were plated in 50 mL bottles and treated with 10^{-6} mol/L of 9-cis RA for 72 h. Cell cycle analysis was carried out using flow cytometry. The results in Figure 6 showed that the cells treated with 9-cis RA had more large populations of cells in G_1 and fewer populations of cells in G_2/M, when comparing with the cells without 9-cis RA treatment. The results indicated that 9-cis RA treatment can cause accumulation of cells in the G_1 phase of the cell cycle and that

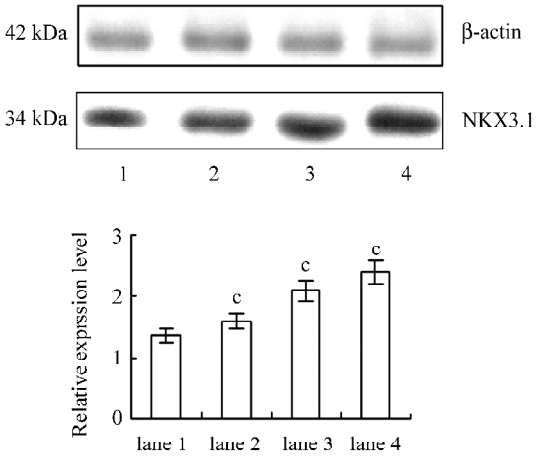

Figure 5. Effect of the 9-cis retinoic acid (RA) treatment on NKX3.1 protein expression in dose-dependent manner. 1, control without the treatment of 9-cis RA; 2, with 10^{-7} mol/L of 9-cis RA treatment; 3, with 10^{-6} mol/L of 9-cis RA treatment; 4, with 10^{-5} mol/L of 9-cis RA treatment. The relative expression levels are presented as the ratio of densities of NKX3.1 and β-actin bands. The results are expressed as mean ± SD ($n = 3$). °$P < 0.01$, the lanes 2, 3, 4 *vs.* lane 1 (control).

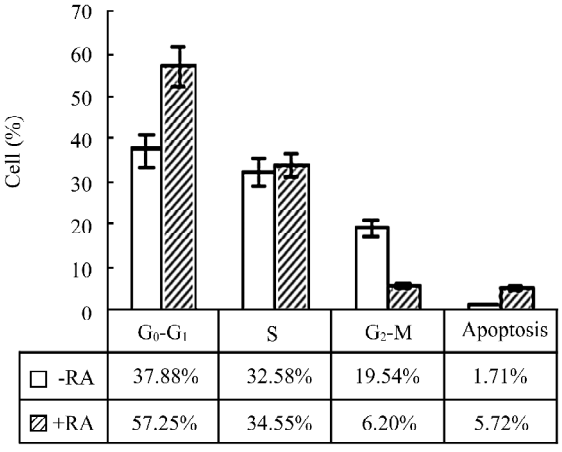

Figure 6. Effects of 9-cis retinoic acid (RA) treatments on cell cycle distribution of LNCaP cells. The results presented as the percentage of the cell distributions in different phases of cell cycle. –RA: cells received dimethyl sulfoxide vehicle and without 9-cis RA treatment. +RA: cells with 10^{-6} mol/L of 9-cis RA treatment. The results were expressed as mean ± SD from triplicate. $P < 0.01$, +RA vs. –RA in G_0-G_1, G_2-M and Apoptosis group; $P > 0.05$, +RA *vs.* –RA in S phase group.

fewer cells pass through to G_2/M. The apoptosis of the cell was increased from 1.71 to 5.72% in assay of flow cytometry.

4 Discussion

The retinoid 9-cis RA has been reported to inhibit the growth of human prostate cancer cell line LNCaP [9]. Our experiments here also demonstrate similar effects of cell cycle arrest and induction of apoptosis in LNCaP cells. However, the precise mechanisms of the effects are still uncertain. It probably exerts its effect by modulating the transcription of a variety of genes critical to cellular proliferation and differentiation. In the present study, we examine the effect of 9-cis RA on regulation of *NKX3.1* gene expression in LNCaP cells that were selected because they were androgen-regulated cell line expressing retinoic acid receptor (RAR) and retinoid X receptor (RXR) [9] as well as *NKX3.1* gene [5]. *NKX3.1* is an androgen regulated prostate-specific homeobox gene that is thought to play an important role in prostate development and cancerogenesis. Previous studies show that over-expression of exogenous *NKX3.1* suppresses growth and tumorigenicity in human prostate carcinoma cell lines [3]. Jia *et al.* [19] demonstrate that *NKX3.1* can lead to prostate cancer cell arrested in the G1 phase of the cell cycle and growth inhibition of the prostate cancer cell line. Our previous studies show that *NKX3.1* overexpression could increase the cell apoptosis in prostate cancer cell line LNCaP (unpublished data). All the above results provide evidence that *NKX3*.1 is a potential growth repressor for prostate cancer cells, and it is possible that *NKX3.1* is one of the downstream genes mediating the growth inhibition effect of 9-cis RA on prostate cancer cells.

Our data shows that the expression of *NKX3.1* gene at both mRNA and protein levels was enhanced in LNCaP cells by 9-cis RA treatment in a dose-dependent manner in RT-PCR and western blot analysis. To identify a regulatory region within the *NKX3.1* promoter contributing to the regulation induced by 9-cis RA treatment, we have constructed the pGL_3-1040bp *NKX3.1* promoter and its 5'-deletion mutants, which were transfected into LNCaP cells with treatment of 9-cis RA. We found in luciferase reporter assay that the region between –936 and –921 bp upstream of *NKX3.1* gene involved the inducible regulation by 9-cis RA treatment. This 16 bp region (RAie) between –936 and –921 has no RA response element according to the database TRANSFAC. Therefore, this might suggest that 9-cis RA interacted indirectly with the 16 bp RAie in *NKX3.1* promoter. Further studies are needed to explain the regulatory mechanism.

In summary, our results demonstrate that 9-cis RA as a differentiating agent can upregulate the expression of human homeobox gene *NKX3.1* that is thought to play an important role in prostate differentiation and to act as a tumor suppressor gene in prostate cancer. The strong association of *NKX3.1* with prostate and prostate cancer development makes this gene an attractive molecular target for intervention and investigation in the field of prostate cancer.

Acknowledgment

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