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p53 overexpression represses androgen-mediated induction of NKX3.1 in a prostate cancer cell line

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Abbreviations: AR, androgen acceptor; ARE, androgen responsive element; CCS, charcoal-treated fetus cattle serum; EMSA, Electrophoresis mobility shift assay; p53 RE, p53 response element; p53mt, mutation type p53; p53wt, wild type p53; PIN, prostatic intraepithelial neoplasia

Abstract

Prostate cancer is a disease involving complicated multiple-gene alterations. Both *NKX3.1* and *p53* are related to prostate cancer and play crucial roles in prostate cancer progression. However, little is known about the relationships and interactions between *p53* and *NKX3.1* in prostate cancer. We found that *NKX3.1* expression is down-regulated by over-expression of wild type (wt) p53 in prostate cancer LNCaP cells. *NKX3.1* is down-regulated at both the mRNA and protein levels by *p53* over-expression due to either transient transfection of exogenous *p53* or induction of endogenous *p53*. *p53* over-expression represses androgen-induced trans-activation of *NKX3.1* by inhibiting the promoter of the androgen acceptor (*AR*) gene and by blocking AR-DNA binding activity. In addition, transfection with the *p21* expression vector (pPSA-*p21*) showed that p21 does not reduce *NKX3.1* expression, indicating that *NKX3.1* expression is not the result of nonspecific effects of cell growth arrest. Our results provide biochemical and cellular biologic evidence that *NKX3.1* is down-regulated by *p53* over-expression in prostate cancer cells.

Keywords: androgens; gene expression regulation; NKX3.1 protein, human; prostate neoplasms; p53

Introduction

NKX3.1 is an androgen regulated prostate-specific homeobox gene (Prescott *et al.*, 1998) that is thought to be involved in prostate development and carcinogenesis (Bhatia-Gaur *et al.*, 1999). Several different mouse models with Nkx3.1 dificiency have demonstrated abnormal ductal morphogenesis, hyperplasias, and PIN-like lesions in the prostate (Abdulkadir *et al.*, 2002; Kim *et al.*, 2002). Human *NKX3.1* has been mapped to human chromosome 8p21, a locus that is frequently deleted in prostate cancer (Cher *et al.*, 1996; Vocke *et al.*, 1996; Dong, 2001; Asatiani *et al.*, 2005). Loss of *NKX3.1* expression has been shown to be associated with hormone-refractory prostate cancer and advanced tumor stage (Bowen *et al.*, 2000); however, over-expression of *NKX3.1* in prostate cancer also has been reported (Xu *et al.*, 2000). No mutations in the *NKX3.1* coding region have been found (Voeller *et al.*, 1997; Ornstein *et al.*, 2001). So far, the function of *NKX3.1* in prostate cancer has been shown to be complicated and the regulatory mechanisms of *NKX3.1* expression are not well known.

The *p53* tumor suppressor gene encodes a nuclear transcription factor that is activated and accumulates in cells in response to a variety of stresses inducing growth arrest or apoptosis. In prostate cancer, alterations in the *p53* gene are clearly associated with a progressive disease, including metastases to bone and androgen-independent growth (Navone *et al.*, 1993; Aprikian *et al.*, 1994; Eastham *et al.*, 1995; Meyers *et al.*, 1998; Burchardt *et al.*, 2001). Recent evidence suggests that p53 is involved in androgen signaling. Cronauer *et al.* analyzed the effect of p53 on androgen signaling in 22Rv1 and LNCaP prostate cancer cells. Overexpression of p53 diminished the androgenic response in both cell lines. *AR*, *p53* and *NKX3.1* are all related to prostate cancer and play crucial roles in prostate cancer progression. However, little is known about their relationships and interactions in prostate cancer. We report that *NKX3.1* expression is regulated by over-expression of wild type (wt) p53 in prostate cancer LNCaP cells and we provide evidence for the mechanisms that control down regulation of *NKX3.1* by p53 over-expression in

Table 1. Primers used in PCR for mutagenesis analysis.

Names	Sequences of primers
PF + 8	5'GGCCTCGAGCGCACCGCTTTCACTTTC3'
PR-1032	5'CGCGAGCTCAAGGCAGGAGGATCACTTG3'
ID-507	5'TGCACCTACTCTTTGAGGTCGTAGATA 3'
ID-518	5'GACCTCAAAGAGTAGGTGCAGCCAGAC 3'

LNCaP cells.

Materials and Methods

Construction of luciferase reporter plasmids

The pGL_3-1040 bp *NKX3.1* promoter was originally constructed by Jiang *et al.* (2004).

There is a 5' half site of the p53 response element (p53 RE) between -517 and -508 upstream of the *NKX3.1* gene. A p53 RE internal deletion from -517 to -508 was performed by a modified inverse PCR method. pGL_3-1040 was used as the template and two pairs of primers (sequences shown in Table 1) were required for inverse PCR. The 5' flanking fragment was amplified using the primers PR-1032 and ID-518, and the 3' flanking fragment was amplified using the primers PF + 8 and ID-507. Inverse PCR was performed using pyrobest polymerase (TaKaRa, Japan) with denaturation at 94°C for 2 min, followed by 35 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min. The amplified 5' and 3' flanking fragments were purified and then ligated by PCR using pyrobest polymerase and the primers PR- 1032 and PF + 8 with denaturation at 94°C for 2 min, annealing at 55°C for 30 s, and extension at 72°C for 2 min, followed by 35 cycles at 94°C for 30 s, 63°C for 30 s, and 72°C for 1 min. The resultant fragment was purified and cut with *Xho*I and *Sac*I, then inserted into the equivalent site of the pGL_3-basic vector (Promega) to generate the p53 RE internal deletion mutant designated as pGL_3-p53id.

Cell culture

The human prostate cancer cell line LNCaP was obtained from the American Type Culture Collection (ATCC). The line, which was established from lymph node metastasis of a prostate cancer patient, expresses the *AR, NKX3.1* and the wild type *p53* genes. LNCaP cells were routinely maintained in RPMI-1640 supplemented with 10% fetal bovine serum (Gibco) and antibiotics. Cells were cultured in RPMI-1640 containing 2% charcoal-treated fetus cattle serum, CCS (Gibco), and supplemented with 10^{-8} mol/l of the synthetic androgen analog R1881 (New England Nuclear, Germany).

Transient transfection

For a luciferase reporter assay, LNCaP cells were seeded in 24-well plates and transfected at approximately 90% cell confluence. pGL_3 constructs were cotransfected with pCMV-*p53* (BD Biosciences, USA), with pCMV-*p53* plus *AR*-expressing plasmid (Dr. Charles Young, Mayo Clinics, USA), with *AR*-expressing plasmid, or with vector lacking cDNA (as a control). The pRL-TK vector (Promega) was used to correct for the transfection efficiency. Transfection was performed using lipofectimine™ 2000 (Invetrigen) according to the manufacturer's instructions. After an incubation period of 6 h in RPMI-1640 without serum and antibiotics, the medium was changed to RPMI-1640 containing 2% CCS, with or without 10^{-8} mol/l of R1881. The reporter activity was assessed after a 48 h incubation period using the Dual-Luciferase Reporter Assay System (Promega, Germany).

For RT-PCR and western blot analysis, LNCaP cells were seeded in 25 ml bottles and transfected with pCMV-*p53*, with pPSA-*p21* (kindly provided by Dr. Charles Young, Mayo Clinics, USA), or with pCMV-*p53* plus *AR*-expressing plasmid. Vectors lacking *p53* cDNA were used as a control. Cells were harvested for RT-PCR and western blot analysis 48 h after transfection.

Dual-luciferase reporter assay

The activities of firefly luciferase in pGL_3 and of Renilla luciferase in pRL-TK were determined following the dual-luciferase reporter assay protocol recommended by Promega. The cells were rinsed twice with PBS buffer and cell lysates were prepared by manually scraping the cells from culture plates in the presence of 1 × PLB (passive lysis buffer). Twenty μl of cell lysate was transferred into a luminometer tube containing 100 μl of LAR II, and the *firefly* luciferase activity (M1) was measured first. The *Renilla* luciferase activity (M2) was then measured after adding 100 μl of Stop & Glo Reagent. The results were calculated and expressed as the ratio of M1/M2. Analyses were carried out twice or three times with double replicates. Data are presented as mean ± S.D.

Electrophoresis mobility shift assay (EMSA)

Nuclear extracts from LNCaP cells were prepared according to the instructions for the nuclear extract kit (ACTIVE MOTIF) and quantified following the BCA method. Double-stranded ARE probes (sense

sequence TGCAGAACAGCAAGTGCTAGC) were generated by annealing equimolar complementary oligonucleotides in TEN buffers at 95°C for 10 min with a slow cool-down period to room temperature. The double-stranded probes were labeled with digoxigenin (Roche, Germany) using terminal transferase (Roche, Germany). For EMSA, 32 pmol of labeled probes were incubated for 20 min at 25°C with 20 μg of nuclear extract in the presence of 1 μg of poly (dI-dC), 0.1 μg of poly L-lysine, 30 mmol/l KCl, 20 mmol/l HEPES, 10 mmol/l $(NH_4)_2SO_4$, 1 mmol/l DTT, 0.2% v/v Tween 20, and 1 mmol/l EDTA. Competition analyses were performed using EMSA conditions similar to those described above, except that the protein extracts were incubated with the probe in the presence of a 125-fold molar excess of unlabeled double-stranded oligonucleotides as a competitor. Subsequently, the DNA-protein complexes were separated from the free probes by electrophoresis on 5% non-denaturing polyacrylamide gel (Sigma). Electro-blotting and chemiluminescent detection were performed according to the instructions for the DIG Gel Shift Kit (Roche, Germany).

Reverse transcription-PCR

Total RNA was extracted from LNCaP cells using

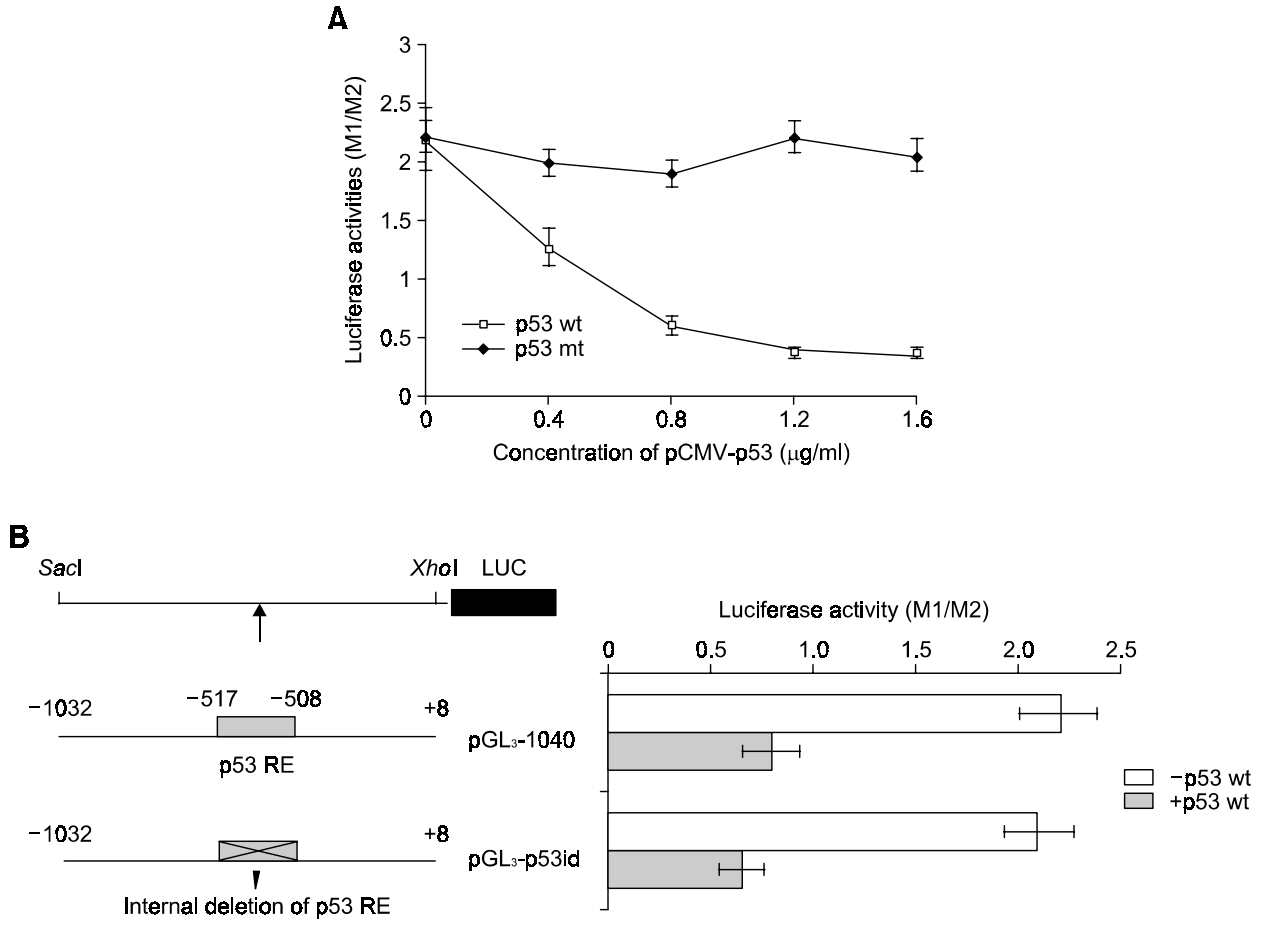

Figure 1. Effect of pCMV-*p53* overexpression on *NKX3.1* promoter activity. (A) LNCaP cells were seeded in 24-well plates and transfected with 0.8 μg of pGL_3-1040 and the indicated concentrations (0-1.6 μg/ml) of either pCMV-*p53* wt or pCMV-*p53* mt. 48 h after transfection, the cells were harvested for a dual-luciferase assay. pRL-TK vector was used to correct for the transfection efficiency. Results are expressed as the ratio of *firefly* luciferase activity (M1) in the pGL_3 plasmid to *Renilla* luciferase activity (M2) in the pRL-TK plasmid. Data are presented as the mean of six individual values ± SD. (B) PCR methods were used to construct the pGL_3-1040bp *NKX3.1* promoter and its internal deletion mutant of p53 RE. PCR produced fragments were separated and purified, then cut with both *Xho*I and *Sac*I and inserted into the equivalent site on the pGL_3-basic vector to generate the constructs designated as pGL_3-1040 and pGL_3-p53id. In the figure, p53 RE donates the p53 response element, and LUC donates the luciferase reporter gene. Either pGL_3-1040 or pGL_3-p53id was co-transfected into LNCaP with, respectively, pCMV-*p53* wt (+ p53 wt) or a vector lacking *p53* cDNA (-p53 wt). 48 h after transfection, the cells were harvested for a dual-luciferase assay. Results are expressed as a relative luciferase activity (M1/M2). Data are presented as the mean of six individual values ± SD.

TRIzol reagent (MBI) following the manufacturer's instructions and expression of *NKX3.1* mRNA was determined using RT-PCR with M-MuL V reverse transcriptase (Invetrigen) in the presence of a random hexamer primer. PCR primers for *NKX3.1* were 5'GTACCTGTCGGCCCCTGAACG3' (sense) and 3'GGACCAGAGGCACATATTGTCG5' (antisense). PCR primers for p53 were 5'TGTCATGGCGACTGTCCAGC3' (sense) and 5'GCTCGACGCTAGGATCTGAC3' (antisense). PCR conditions were denaturation at 94°C for 2 min, followed by 25 cycles at 94°C for 30 s, 63°C (for NKX3.1) or 58°C (for p53) for 30 s, and 72°C for 40 s, followed by heating at 72°C for 8 min. A 550 bp segment of *β-actin* mRNA was amplified and used to normalize the quantity of the *NKX3.1* mRNA in RT-PCR.

SDS-polyacrylamide gel electrophoresis and western blot analysis

LNCaP cells were harvested and lysed with cell lyses buffer (50 mmol/l Tris·HCl, pH 8.0, 150 mmol/l NaCl, 0.1% SDS, 100 μg/ml of PMSF, 1 μg/ml of aprotinin, and 1% NP-40). Cell extracts were quantified according to the BCA method. For Western blot analysis, 30 μg of cell extract was separated on 10% SDS-PAGE, then electroblotted onto nitrocellulose membrane. After blocking and washing, the membrane was exposed to specific anti-human NKX3.1 antibody (Research Diagnostics Inc) at 4°C for 12 h, followed by incubation with peroxidase-labeled second antibody (Sigma) for 1 h at room temperature. Immunoreactive bands were visualized *via* enhanced chemiluminescence (Santa Cruz Biotech. Inc). β-tubulin (Sigma) was used to normalize the quantity of the protein on the blot.

Results

Construction of luciferase reporter plasmids

To observe the effects of p53 on *NKX3.1* promoter activity, we constructed the pGL_3-1040 bp *NKX3.1* promoter and its internal deletion mutant of a potential p53 response element (p53 RE). PCR methods were used in the construction and the related primers are listed in Table 1. A schematic depiction of the construction is shown in Figure 1B. All constructs were confirmed to be correct by restriction enzyme digestion and sequence analysis. pGL_3-p53id with p53 RE deleted from -517 to -508 showed no effect on *NKX3.1* promoter activity (blank bar of pGL3-p53id vs. blank bar of pGL3-1040 in Figure 1B). However, the p53 inducible negative effect remained (grey bar of pGL3-p53id in Figure 1B), indicating that this p53 RE is probably not a functional

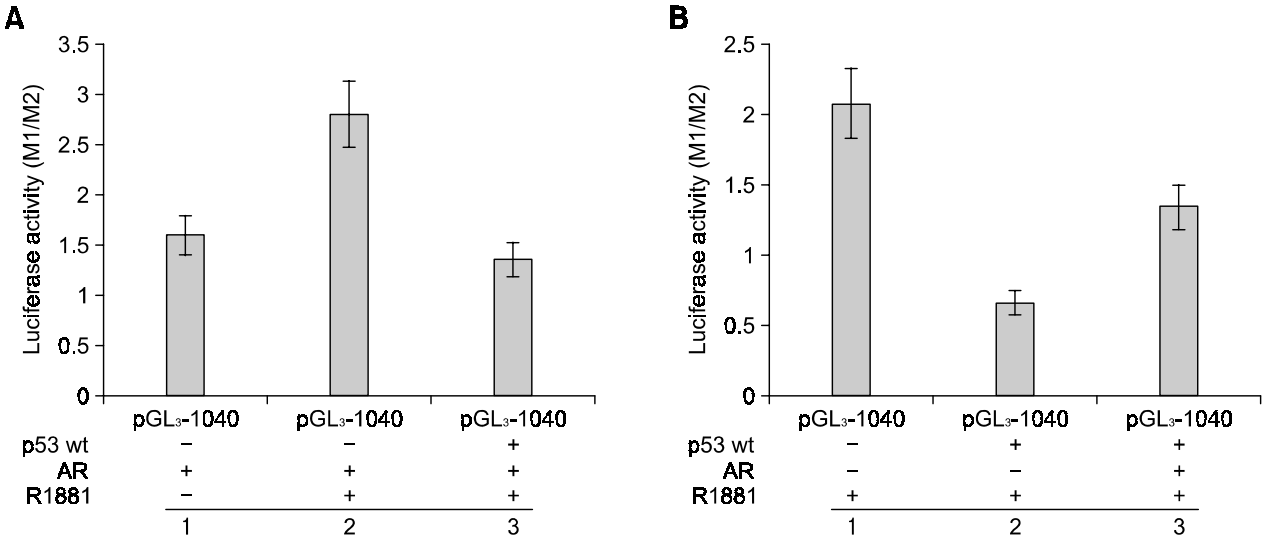

Figure 2. *p53* over-expression repressed androgen-induced transactivation of the *NKX3.1* promoter. LNCaP cells were seeded in 24-well plates with a 2% CCS medium for a luciferase reporter assay. (A) 0.6 μg of pGL_3-1040 was transfected into LNCaP cells with either 0.6 μg/ml of *AR*-expressing plasmid both without R1881 treatment (bar 1) and with R1881 (10^{-8} mol/L) treatment (bar 2), or with 0.6 μg of *AR*-expressing plasmid plus 0.6 μg/ml of pCMV-*p53* wt with R1881 treatment (bar 3). (B) 0.6 μg of pGL_3-1040 was transfected into LNCaP cells with R1881 (10^{-8} mol/L) treatment (bar 1), with 0.6 μg/ml of pCMV-p53 wt and R1881 (10^{-8} mol/L) treatment (bar 2), or with 0.6 μg of AR-expressing plasmid plus 0.6 μg/ml of pCMV-*p53* wt with R1881 (10^{-8} mol/L) treatment (bar 3). Results are expressed as the luciferase activity (M1/M2). Data are presented as the mean of four individual values ± SD.

element in a LNCaP prostate cancer cell line.

Inhibition of *NKX3.1* promoter activity by over-expression of wild p53

To observe the effects of p53 on *NKX3.1* promoter activity, LNCaP cells were seeded in 24-well plates and co-transfected with 0.8 μg of pGL_3-1040 and either 0-1.6 μg/ml of pCMV-*p53* wt or pCMV-*p53* mt (BD Biosciences) using lipofectimine™ 2000. Cells were harvested for a dual-luciferase assay 48 h after transfection. The results (Figure 1A) showed that wild type p53 (p53 wt) and not mutant type p53 (p53 mt) reduced 1040 bp-*NKX3.1* promoter activity in a dose-dependent manner.

p53 over-expression repressed androgen-induced transactivation of *NKX3.1*

There is a 5' half site of the p53 response element (p53 RE) between -517 and -508 upstream of the *NKX3.1* gene. However, the p53 RE internal deletion showed no effect on *NKX3.1* promoter activity, indicating that induction of NKX3.1 is not directly regulated by p53 binding. *NKX3.1* is an androgen-regulated homeobox gene, the expression of which can be regulated by androgen and androgen receptor activity. To investigate whether this inducible negative effect of p53 on *NKX3.1* promoter is related to AR activity, pGL_3-1040 was co-transfected into LNCaP cells with pCMV-*p53* wt and an *AR*-expressing vector. The results (Figure 2) showed that *p53* over-expression repressed androgen-induced transactivation of the *NKX3.1* promoter (bar 3 in Figure 2A), and that AR overexpression can relieve the negative effect of p53 on NKX3.1 promoter activity (bar 3 in Figure 2B), indicating that AR is probably involved in the negative effect of p53 on the *NKX3.1* promoter and that p53 represses androgen-induced transactivation of *NKX3.1*.

To detect the effect of p53 over-expression on *AR* gene promoter activity, pCMV-p53 wt was co-transfected into LNCaP cells with the pGL_3-*AR* promoter (supplied by Dr. Charles Young, Mayo Clinic) using lipofectimine™ 2000. The result (Figure 3) showed that *AR* promoter activity was reduced by p53 over-expression in a dose-dependent manner based in a luciferase reporter assay.

To detect the effect of *p53* over-expression on AR binding activity with ARE, EMSA was performed using a DIG-labeled ARE probe and nuclear extracts from LNCaP cells that were transfected with pCMV-*p53* wt. The result (Figure 4) showed that *p53* over-expression inhibited the AR binding activity with

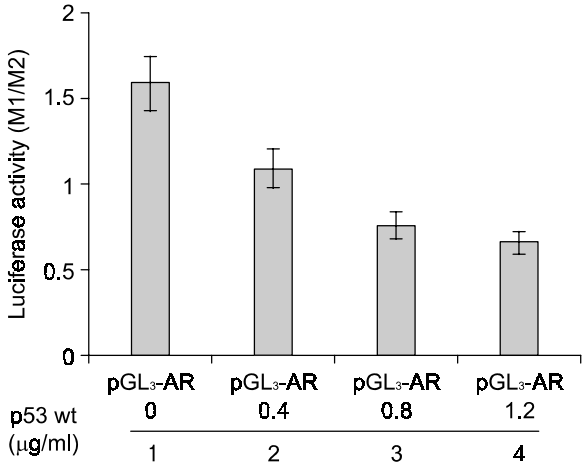

Figure 3. Inhibition of *AR* promoter activity by *p53* over-expression. LNCaP cells were seeded in 24-well plates and co-transfected with 0.8 μg of the *AR* promoter-reporter plasmid (pGL_3-AR) and 0, 0.4, 0.8, or 1.2 μg/ml (the grey bars from 1-4) of pCMV-*p53* wt treated with 10^{-8} mol/l of R1881. 48 h after transfection the cells were harvested for a luciferase reporter assay. Results are expressed as the relative luciferase activity (M1/M2). Data are presented as the mean of four individual values ± SD.

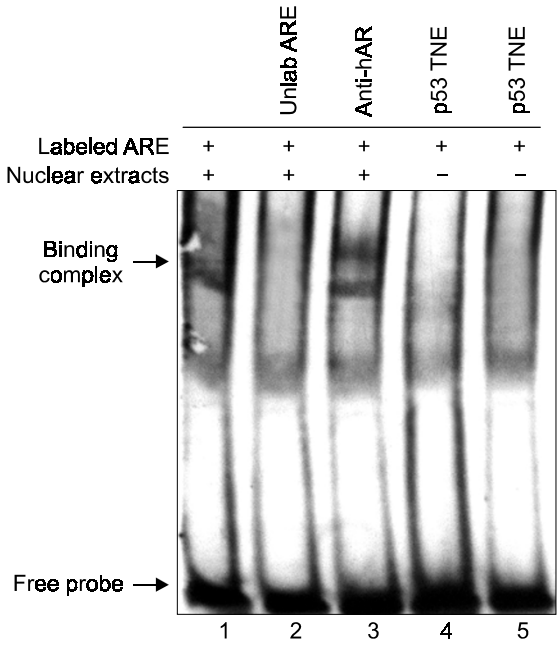

Figure 4. Assay of AR binding activity with ARE in EMSA. LNCaP cells were transfected with 1.0 μg/ml of *AR*-expressing plasmid either with or without pCMV-*p53* wt, then treated with 10^{-8} mol/l of R1881. Nuclear extracts from LNCaP cells were tested for AR DNA binding in EMSA. 32 pmol of DIG-labeled ARE was incubated with 20 μg of nuclear extract from cells transfected with 1.0 μg/ml of *AR*-expressing plasmid plus either 0.8 μg/ml of the pCMV plasmid lacking cDNA (lane 1-3), or 0.8 μg/ml of pCMV-*p53* wt (lane 4), or 1.2 μg/ml of pCMV-*p53* wt (lane 5). Competition was carried out with a 125-fold excess of unlabeled ARE (lane 2). Anti-hAR was used in supershift (lane 3). Note that p53 TNE denotes p53 treated nuclear extracts. ARE represents the androgen response element.

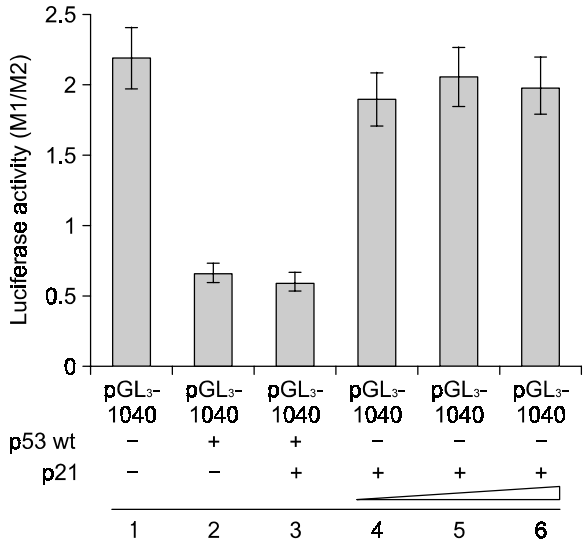

Figure 5. Effects of p21 on *NKX3.1* promoter activity. LNCaP cells were seeded in 24-well plates and co-transfected with 0.8 μg of pGL_3-1040 and 0.8 μg/ml of pCMV lacking cDNA (bar 1), with 0.8 μg of pGL_3-1040 and 0.8 μg/ml of pCMV-*p53* wt (bar 2), with 0.8 μg of pGL_3-1040 and 0.8 μg/ml of pCMV-*p53* wt plus 0.4 μg/ml of pPSA-*p21* (bar 3), or with 0.8 μg of pGL_3-1040 and 0.4, 0.8, 1.2 μg/ml of pPSA-*p21* without pCMV-*p53* (bars 4-6). After a 48 h incubation period with RPMI-1640 containing 2% CCS and 10^{-8} mol/l of R1881, the cells were harvested for a luciferase reporter assay. Results are expressed as luciferase activities (M1/M2). Data are presented as the mean of four individual values ± SD.

ARE (lane 4, 5 in Figure 4).

p53 over-expression probably repressed androgen-induced transactivation of *NKX3.1* by inhibiting the promoter of the *AR* gene and by blocking AR-DNA binding activity.

Down-regulation of *NKX3.1* by *p53* over-expression is not mediated by p21 and is not a result of cell growth arrest

p53 expression can lead to induction of the downstream target gene $p21^{waf1/cip1}$ that is an inhibitor of the cell cycle and that causes cell growth arrest. To demonstrate whether *NKX3.1* down-regulation is mediated by this p53-p21 pathway, LNCaP cells were co-transfected with pGL_3-1040 and a *p21*-expressing vector (pPSA-*p21*). The results (Figure 5) showed that p21 did not reduce *NKX3.1* promoter activity (bars 4-6). Only p53 reduced *NKX3.1* promoter activity (bars 2, 3). These results indicate that the *NKX3.1* promoter was probably specifically inhibited by *p53* over-expression and was not inhibited as a result of cell growth arrest.

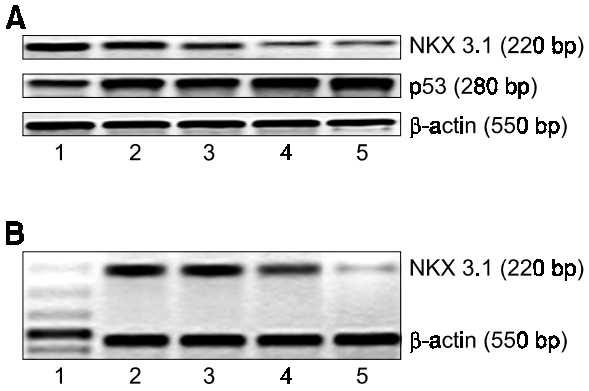

Figure 6. Effects of *p53* over-expression on *NKX3.1* mRNA expression in LNCaP cells. LNCaP cells were transfected with the indicated plasmids for 48 h. RT-PCR was carried out to analyze *NKX3.1* mRNA expression. (A) 1, control with pCMV lacking cDNA transfection; 2-5, transfection with 0.5, 1.0, 2.0, or 4.0 μg/ml of pCMV-*p53* wt; (B) 1, DNA marker; 2, control with pCMV lacking cDNA transfection; 3, transfection with 4.0 μg/ml of pPSA-*p21*; 4, transfection with 2.0 μg/ml of pCMV-*p53* wt plus 2.0 μg/ml of *AR*-expressing plasmid; 5, treatment with UV irradiation. β-actin was used as an internal control.

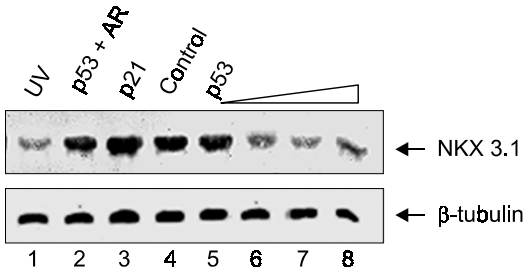

Figure 7. Effects of *p53* over-expression on NKX3.1 protein expression in LNCaP cells. LNCaP cells were transfected with the indicated plasmids for 48 h. Western blot analysis was carried out to analyze NKX3.1 protein expression. Lane 1, treatment with UV irradiation; 2, transfection with 2.0 μg/ml of pCMV-*p53* wt plus 2.0 μg/ml of *AR*-expressing plasmid; 3, transfection with 4.0 μg/ml of pPSA-*p21*; 4, control transfected with pCMV lacking the cDNA vector; 5-8, transfection with 0.5, 1.0, 2.0, or 4.0 μg/ml of pCMV-p53 wt. β-tubulin was used as an internal control for protein loading and transfer efficiency.

p53 over-expression down-regulates androgen-mediated *NKX3.1* expression

RT-PCR and a Western blot assay were performed to investigate the effects of *p53* over-expression on *NKX3.1* expression. LNCaP cells were cultured in 25 ml bottles and treated with 0.5-4.0 μg/ml of pCMV-*p53* wt (lanes 2-5 in Figure 6A and lanes 5-8 in Figure 7), with 4.0 μg/ml of pPSA-*P21* plasmid (lane 3 in Figure 6B and lane 3 in Figure 7), or with 2.0 μg/ml of pCMV-*p53* wt plus 2.0 μg/ml of *AR*-expressing plasmid (lane 4 in Figure 6B and lane 2 in Figure

7). In addition, LNCaP cells were exposed to UV irradiation to induce endogenous *p53* expression (lane 5 in Figure 6B and lane 1 in Figure 7). As shown in Figures 6 and 7, the expressions of *NKX3.1* mRNA and protein were down-regulated by *p53* over-expression in a dose-dependent manner. Endogenous p53 induced by UV irradiation also inhibited *NKX3.1* mRNA and protein expression. To determine whether p21 and AR are involved in p53 negative regulation of *NKX3.1*, LNCaP cells were treated with pPSA-*p21*, or with pCMV-*p53* wt plus *AR*-expressing plasmid. Results showed that p21 did not down-regulate *NKX3.1* expression (lane 3 in Figure 6B and lane 3 in Figure 7) while AR relieved p53 inhibition of *NKX3.1* expression (lane 4 in Figure 6B and lane 2 in Figure 7). These results indicate that *NKX3.1* expression is probably specifically reduced by p53 and mediated by AR, and is not the result of nonspecific effects of the cell growth arrest mediated by p21.

Discussion

p53 is a transcription factor that recognizes a specific consensus DNA sequence consisting of two copies of a 10-bp motif, 5'-PuPuPuC(A/T)(T/A)GPy-PyPy- 3'. Wild-type p53 (but not mutants) efficiently binds to this sequence and transactivates expression of the target genes (El-Deiry *et al.*, 1992; 1993; Kastan *et al.*, 1992; Okamoto *et al.*, 1994; Miyashita *et al.*, 1995). p53 can also repress a wide variety of cellular and viral promoters that lack p53 binding sites, including *c-fos*, *bcl-2*, and the insulin-like growth factor I receptor (Santhanam *et al.*, 1991; Kley *et al.*, 1992; Subler *et al.*, 1992; Donehower *et al.*, 1993; Miyashita *et al.*, 1994; Werner *et al.*, 1996). Furthermore, p53 can bind to the TATA-binding protein and repress promoter activity (Seto *et al.*, 1992; Liu *et al.*, 1993). p53 also interacts with other transcription factors, including Sp1 (Borellini *et al.*, 1993) , the CCAAT-binding factor (Agoff *et al.*, 1993), the cAMP response element-binding protein (Desdouets *et al.*, 1996), and glucocorticoid receptors (Maiyar *et al.*, 1997). These observations indicate that p53 interacts with the transcription machinery to modulate gene expression. We found that *NKX3.1* expression is down-regulated by over-expression of wild type *p53*. *NKX3.1* is down-regulated at both the mRNA and protein levels by *p53* over-expression due to either transient transfection of exogenous *p53* or induction of endogenous *p53* in LNCaP cells.

There is increasing evidence that p53 directly regulates androgen signaling. The p53 protein interacts with several steroid receptors, including AR (Yu *et al.*, 1997a; 1997b; Sengupta *et al.*, 2001; Shenk *et al.*, 2001). AR is a member of the nuclear receptor superfamily of transcription factors (Evans, 1988; O'Malley, 1990) and is activated either by its androgen ligand or in a ligand-independent manner (Brinkmann *et al.*, 1999; Feldman and Feldman, 2001; Grossmann *et al.*, 2001; Culig, 2004). Subsequently, the activated receptor homodimerizes and interacts with specific androgen response elements in the regulatory regions of androgen target genes, resulting in stimulation of gene expression. Over-expression of *p53* down-regulates the androgenic response in prostate cancer cells (Cronauer *et al.*, 2004) and p53 blocks AR DNA binding by disrupting the amino- to carboxyl-terminal (N-to-C) interaction, which is thought to be responsible for the homodimerization of this receptor (Shenk *et al.*, 2001). Over-expression of *p53* in LNCaP cells probably results in a dramatic decrease in AR-transactivation of the androgen-inducible gene. *NKX3.1* is an androgen-regulated homeobox gene in the prostate, the expression of which can be regulated by androgen and androgen receptor activity. We found that *p53* over-expression represses androgen-induced transactivation of *NKX3.1* (Figure 2) by inhibiting the *AR* promoter (Figure 3) and by blocking AR-DNA binding activity (Figure 4), indicating that AR is probably involved in the negative effects of p53 on the *NKX3.1* promoter.

There is a potential p53 RE between -517 and -508 upstream of the *NKX3.1* gene shown in the TRANSFAC database. To determine whether this p53 RE is a functional element for mediation of p53 inducible regulation of the *NKX3.1* promoter, we constructed a mutant (pGL$_3$-p53id) of the *NKX3.1* promoter with p53 RE deleted from -517 to -508 of *NKX3.1* (Figure 1B). Results showed that pGL$_3$-p53id had no effect on *NKX3.1* promoter activity and still retained a p53 inducible negative effect on the NKX3.1 promoter (Figure 1B), indicating that this p53 RE is not a functional element in the LNCaP prostate cancer cell line.

DNA-damaging agents are inducers of *p53* expression that lead to induction of downstream target genes, such as $p21^{waf1/cip1}$, which encode a G_1 cyclin/CDK inhibitor that causes cell growth arrest (Levine 1997). To determine whether *NKX3.1* down-regulation is mediated by this p53-p21 pathway, LNCaP cells were transfected with either *p53*wt or the *p21* expressing vector pPSA-*p21*. *p21* transfection had no effect on *NKX3.1* promoter activity (Figure 5) and *NKX3.1* expression (Figures 6 and 7), indicating that *NKX3.1* was specifically inhibited by *p53* over-expression and not by cell growth arrest. In addition, to determine whether *NKX3.1* expression is inducible in response to DNA damage, LNCaP cells

were UV-irradiated and the effects of endogenous p53 on *NKX3.1* expression were analyzed. As shown in Figures 6 and 7, UV irradiation treatment reduced *NKX3.1* expression in LNCaP cells containing endogenous wild type p53.

It would be interesting to investigate the mechanism for indirect-repression of the *NKX3.1* gene by p53. Our data show that *NKX3.1* expression is down-regulated at both the mRNA and protein levels by *p53* over-expression in LNCaP cells. The negative effects of p53 on *NKX3.1* may be related to *p53* over-expression repressing androgen-induced transactivation of *NKX3.1* by inhibiting the promoter of the androgen acceptor gene, and by blocking of the AR-DNA binding activity. Furthermore, this negative effect of p53 on *NKX3.1* is not the result of cell growth arrest mediated by p21. Both *NKX3.1* and *p53* are related to prostate cancer and play crucial roles in prostate cancer progression. In prostate cancer, alterations in the *p53* tumor suppressor gene are clearly associated with a progressive disease. Loss of *NKX3.1* expression (Bowen *et al.*, 2000) or *NKX3.1* over-expression (Xu *et al.*, 2000) has been shown to be associated with hormone-refractory prostate cancer and advanced tumor stage. Further molecular biological and clinical investigations will be needed to interpret the relationship between *p53* and *NKX3.1* in prostate cancer.

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