Blackwell Publishing

Asian J Androl 2007; 9 (3): 353–360
DOI: 10.1111/J.1745-7262.2007.00278.X

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·*Original Article*·

Expression of *Nkx3.1* enhances 17β-estradiol anti-tumor action in PC3 human prostate cancer cells

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Abstract

Aim: To explore whether the anti-tumor action of 17β-estradiol is enhanced by re-expression of the homeodomain transcription factor *Nkx3.1* in PC3 human prostate cancer cells. **Methods:** PC3 cells were stably transfected with pcDNA3.1-Nkx3.1-His vector, which carries a full-length cDNA of human *Nkx3.1*. The PC3 cells stably transfected with vector pcDNA3.1 were set as a control. The expression of Nkx3.1 protein in the cells was confirmed by Western blot analysis. The effect of *Nkx3.1* on cell proliferation of PC3 cells was examined with MTT assay. The antiproliferative and apoptotic effects of 17β-estradiol alone or in combination with *Nkx3.1* were estimated on PC3 cells by using MTT growth tests and flow cytometric analyses. The expression of apoptosis-related proteins was analyzed using Western blotting. **Results:** The plasmid carrying *Nkx3.1* gene induced high expression of Nkx3.1 protein in PC3 cells. The re-expression of exogenous Nkx3.1 did not cause a significant reduction in cellular proliferation, whereas the expression of *Nkx3.1* enhanced the 17β-estradiol anti-proliferative effect in PC3 cells. Nkx3.1 expression promoted 17β-estradiol-induced apoptosis of PC3 cells, as shown by analysis of Bcl-2, Bax, Caspase-3 and poly (ADP-ribose) polymerase expression. **Conclusion:** The present study demonstrates that re-expression of *Nkx3.1* enhances 17β-estradiol anti-tumor action in PC3 human prostate cancer cells. The *in vitro* study suggests that re-expression of *Nkx3.1* is worthy of further consideration as an adjuvant treatment of androgen independent prostate cancer with estrogen anti-tumor therapies. *(Asian J Androl 2007 May; 9: 353–360)*

Keywords: apoptosis; estrogen; *Nkx3.1*; prostate cancer cell; 17β-estradiol; androgen independent prostate cancer

1 Introduction

Initial treatment for patients with advanced or recurrent prostate cancer is typically surgical or medical castration: androgen deprivation therapy (ADT). Although most cases initially respond to androgen deprivation, eventually this therapy fails and the patient dies of recurrent androgen-independent prostate cancer (AIPC). The lack of effective therapies for AIPC continues to spur new efforts to find therapeutic avenues for managing this prevalent male neoplasm.

Estrogen therapies, such as diethylstilbesterol (DES), PC-SPES, transdermal estradiol and conjugated estrogens, are effective in the treatment of AIPC [1, 2]. Oral estrogen treatment with DES, once the most common method

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Received 2006-06-03 Accepted 2007-03-10

for hormone manipulation of prostate cancer, was largely abandoned in the 1970s because of its significant thromboembolic and cardiovascular toxicity [3]. However, in the mid-1980s, interest in estrogen therapy was renewed when it was found that estrogen given parenterally did not induce such side effects [3, 4]. A phase II study of transdermal estradiol in patients with AIPC demonstrated that estradiol was well tolerated and produced a modest response rate, but was not associated with thromboembolic complications or clinically important changes in several coagulation factors [2].

Nkx3.1 is a prostate-specific homeobox gene that is located on chromosome 8p21 [5]. Null alleles of *Nkx3.1* in mice results in impaired prostate development as well as hyperplasia and dysplasia of the prostate. In addition, the *Nkx3.1* gene maps to a region of high loss of heterozygosity in prostate cancer in humans, suggesting that *Nkx3.1* might have a direct role in prostate carcinogenesis, possibly functioning as a tumor suppressor protein [6, 7]. Loss of *Nkx3.1* expression is strongly associated with hormone-refractory disease and advanced tumor stage in prostate cancer [8]. In the LNCaP androgen-dependent prostate cancer cell line, *Nkx3.1* is expressed at a basal level that increases upon androgen stimulation. In contrast, there is no *Nkx3.1* expression in androgen-independent PC3 cells [9]. In addition to androgens, *Nkx3.1* expression is upregulated by 17β-estradiol [10]. Based on the results of the abovementioned studies, we hypothesized that the re-expression of *Nkx3.1* would enhance estradiol anti-tumor action in PC3 human prostate cancer cells.

The objective of the present study is to investigate whether the anti-tumor action induced by estradiol could be enhanced by the restoration of *Nkx3.1* expression in PC3 cells.

2 Materials and methods

2.1 Cell culture

PC3 and LNCaP, derived from human prostate cancer, were obtained from the American Type Culture Collection (Rockville, MD, USA). The cells were maintained in RPMI-1640 medium (Hyclone, Utah, USA) with 10% fetal bovine serum at 37°C in a humidified atmosphere with 5% CO_2.

2.2 Expression and reporter plasmid construction

Total RNA was isolated from normal prostate tissue using TRIzol (Invitrogen, California, USA) according to the manufacturer's instructions. Two micrograms of total RNA was used for cDNA synthesis (SuperScript III Reverse Transcriptase; Invitrogen, California, USA) and one-tenth of the obtained cDNA was used to amplify *Nkx3.1* and *Nkx3.1-HisTag* by polymerase chain reaction (PCR). The conditions of PCR for each individual gene were optimized to analyze amplified product in the linear range of amplification by adjusting amplification cycles for each set of primer. The nucleotide sequences of the primers used to amplify the *Nkx3.1* and *Nkx3.1-HisTag* genes were as follows: *Nkx3.1*, sense primer 5'-CGC GGA TCC GCG ATG CTC AGG GTT CCG GAG C-3' and antisense primer 5'-CCG GAA TTC CGG TTA CCC AAA AGC TGG GCT C-3', *Nkx3.1-HisTag*, sense primer 5'-CGC GGA TCC GCG ATG CTC AGG GTT CCG GAG C-3' and antisense primer 5'-CCG GAA TTC TTA ATG GTG ATG ATG CCC AAA AGC TGG GCT CCA GC-3'.

The amplified *Nkx3.1* and *Nkx3.1-His* DNA samples were cloned into a pGEM-T Easy vector (Promega, Wisconsin, USA), following the protocol provided by the manufacturer. The DNA were digested with *EcoRI* and *BamHI* restriction enzymes and the fragment representing *Nkx3.1* and *Nkx3.1-His* cDNA were excised from a 1% agarose gel. The DNA was purified using a QIAquick Gel extraction Kit (Qiagen, Hilden, Germany). The resulting *Nkx3.1-His* fragment was directionally cloned into the *EcoRI* and *BamHI* restriction sites of plasmid pcDNA3.1 (–) (Invitrogen, California, USA). *Nkx3.1* was cloned into pEGFP-C1 (Clontech, California, USA) at *BglII* and *EcoRI* sites. The successful cloning was confirmed by sequencing the plasmid.

2.3 Stable transfection of PC3 cells

For transfection, plasmid DNA were prepared using a Qiagen plasmid midi kit (Qiagen GmbH, Hilden, Germany). PC3 cells were seeded at a concentration of 5×10^5 cells per well into 6 well culture dishes. Cells were allowed to adhere overnight, and the next day washed twice with serum-free RPMI-1640. Transfection solution containing Lipofectamine 2000 (Invitrogen, California, USA) and DNA (pcDNA3.1, pcDNA3.1-Nkx3.1-His, GFP-Nkx3.1) was carefully overlaid and incubated with the cells for 5 h. RPMI-1640 with 10% fetal bovine serum was next added and the cells were incubated for 2 days, with media being replaced every day. On the third day, medium was replaced with selection medium containing 500 μg/mL of G418. Stable clones were iso-

lated using cloning cylinders and grown continuously under G418 selection pressure.

2.4 Proliferation assay

To analyze the effect of *Nkx3.1* on cell growth, MTT assay (CellTiter 96 nonradioactive cell proliferation assay kit [Promega]) was used to quantify cell proliferation. Nkx3.1-PC3 and pcDNA3.1-PC3 cells were plated in 96 well plates at a density of 5.0×10^3 cells per well containing 100 μL of culture medium. After 0, 24, 48, 72, 96 and 120 h culture, MTT (0.5 mg/mL in phosphate-buffered saline [PBS]) was added to each well and incubated for 4 h at 37°C. The medium was then carefully aspirated, and dimethyl sulfoxide was added to solubilize the colored formazan product. Absorbance value was read on a scanning multiwell spectrophotometer (Bio-Rad, California, USA) with 570 nm wavelength after agitating the plates for 5 min on a shaker. Each experimental condition was performed in six preparations and repeated three times.

Following stable transfection of PC3 cells, the cells were treated with various concentrations of 17β-estradiol (Sigma). Nkx3.1-PC3 and pcDNA3.1-PC3 cells were weaned off steroids in phenol red-free RPMI-1640 supplemented with 10% charcoal dextran-treated fetal calf serum before experiments. Cells were estrogen-depleted for 3 days and then plated in 96 well plates at a density of 5.0×10^3 cells per well and treated with various concentrations of 17β-estradiol. Proliferation assay was performed as described above.

2.5 Flow cytometric analysis of cell apoptosis

Annexin V (Annexin V-FITC) and propidium iodide (PI) double staining was used to determine apoptosis. Annexin V binds to cells that express phosphatidylserine on the outer layer of the cell membrane, and PI stains cellular DNA of cells with a compromised cell membrane. Cells that stain positive for Annexin V-FITC and negative for PI are undergoing apoptosis. Cells that stain positive for both Annexin V-FITC and PI are either in the end stage of apoptosis, or are already dead. Cells that stain negative for both Annexin V-FITC and PI are alive and not undergoing measurable apoptosis. Cells were collected and washed twice with cold PBS and were resuspended in 1× binding buffer at a concentration of 1×10^6 cells/mL. Cells were double stained with FITC-conjugated annexin V and PI for 15 min at room temperature. Annexin V and PI were added according to the manufacturer's recommendations (BD, pharMingen, San Diego, CA, USA). Samples were immediately analyzed by flow cytometry.

2.6 Western blot analysis

Treated cells were washed with cold PBS and lysed in protein extraction buffer (20 mmol/L Tris, pH 8.0, 137 mmol/L NaCl, 10% glycerol, 1% Nonidet P-40, 0.1% SDS, 0.5% sodium dexycholate, 2 mmol/L EDTA) containing protease inhibitors (Cocktail set III, Calbiochem, California, USA). The protein concentration of each sample of the tumor cell lysates was quantified using Coomassie protein assay reagent (Pierce, Rockford, IL, USA). An equal amount of protein was subjected to 10% SDS-PAGE and transferred to polyvinylidene difluoride membrane by blotting overnight at 30V/35 mA. Membranes were blocked for 2 h at room temperature in 5% (w/v) non-fat milk/Tris-buffered saline with 0.1% (v/v) Tween-20. Western blotting was carried out overnight at 4°C using the following antibodies: Nkx3.1 polyclonal antibody (1:200; H-50, Santa Cruz Biotechnology). His-Tag monoclonal antibody (1:1000; 70796-4, Novagen, Darmstadt, USA), Bax monoclonal antibody (1:200; B-9, Santa Cruz Biotechnology, California, USA), Bcl-2 monoclonal antibody (1:200; C-2, Santa Cruz Biotechnology, California, USA), Caspase-3 monoclonal antibody (1:200; E-8, Santa Cruz Biotechnology, California, USA), poly(ADP-ribose) polymerase (PARP) monoclonal antibody (1:200; F-2, Santa Cruz Biotechnology, California, USA) and glyceraldehydes-3-phosphate dehydrogease monoclonal antibody (1:5 000; Acris antibodies GmbH, Hiddenhausen, Germany). Blots were then incubated with 1:2 000 dilution for goat anti-rabbit IgG peroxidase conjugated secondary antibody (Sunnyvale, CA, USA) or 1:2 000 diluation for goat anti-mouse IgG peroxidase conjugated secondary antibody (Santa Cruz Biotechnology, California, USA), washed, and developed by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

2.7 Statistical analysis

For all groups, data are presented as the mean ± SE. Results were analyzed by one-way analysis of variance and Student's *t*-test to identify significant differences between groups. The levels of statistical significance were set at $P < 0.05$, and all statistical calculations were done using SPSS software (version 10.1, SPSS, Chicago, IL, USA).

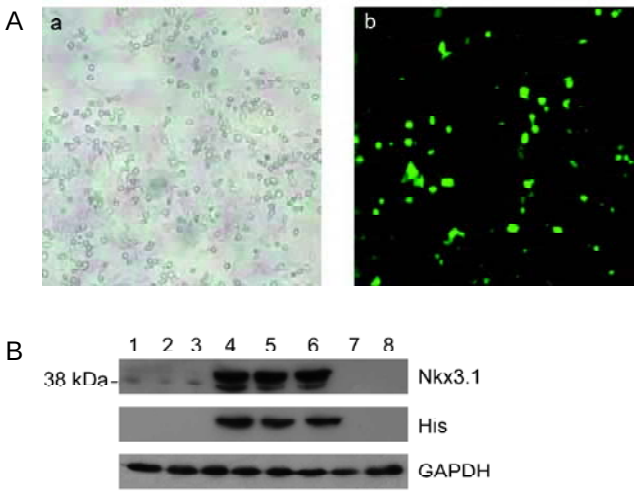

Figure 1. *Nkx3.1* expression in stably transfected PC3 cells. (A): PC3 cells were stably transfected with pEGFP-Nkx3.1, GFP fusion protein expression was monitored by fluorescence microscopy. a, pEGFP-Nkx3.1-PC3 cells under phase contrast microscopy; b, pEGFP-Nkx3.1-PC3 cells under fluorescence microscopy. (B): Content of Nkx3.1 in PC3 cells stably transfected with Nkx3.1-His. Cells were harvested and lysed for Western blot analysis. There was basal level of *Nkx3.1* expression in LNCaP cells. Control pcDNA3.1-PC3 cells and PC3 cells do not show any detectable Nkx3.1, whereas Nkx3.1-His transfected PC3 cells are positive for Nkx3.1 staining. The expression of His is in accordance with Nkx3.1 in transfected PC3 cells. Lanes 1–3, LNCaP cells; lanes 4–6, Nkx3.1-His-PC3 cells; lane 7, pcDNA3.1-PC3 cells; lane 8, PC3 cells. GAPDH, Glyceraldehydes-3-phosphate dehydrogease.

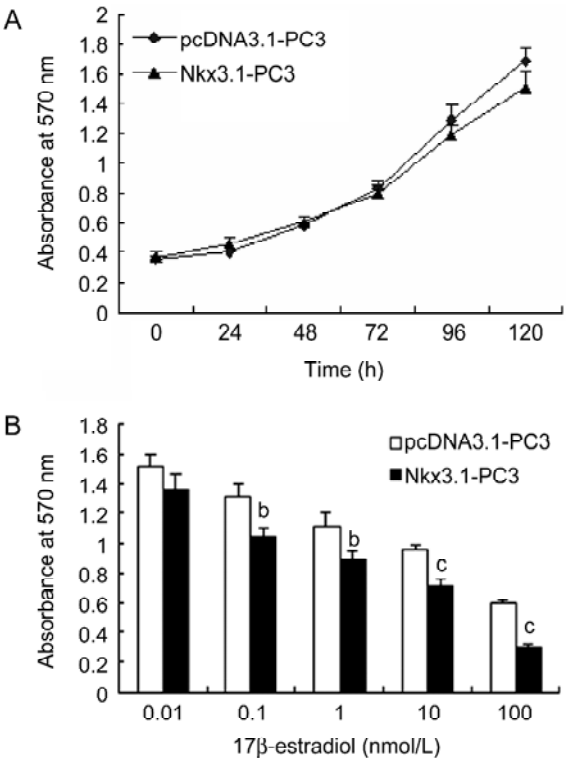

Figure 2. *Nkx3.1* enhances 17β-estradiol anti-proliferative effect in PC3 cells. (A): Nkx3.1-PC3 cells and pcDNA3.1-PC3 cells were plated in 96 well plate and cultured in a 5% CO_2 humid incubator at 37°C. Cell proliferation assay was performed at 0, 24, 48, 72, 96 and 120 h using MTT assay, and was expressed as the value of absorbance at 570 nm. (B): Nkx3.1-PC3 cells and pcDNA3.1-PC3 cells were incubated with 17β-estradiol, from 0.01 nmol/L up to 100 nmol/L. After 5 days of exposure, cell proliferation was analyzed as above. Data shown are mean ± SE (n = 3). $^bP < 0.05$; $^cP < 0.01$, compared with pcDNA3.1-PC3 cells.

3 Results

3.1 Nkx3.1 expression in stably transfected PC3 cells

Following stable transfection of PC3 cells, reporter gene expression was analyzed by fluorescence microscopy. Many brightly fluorescence cells were found in stably transfected Nkx3.1-GFP-PC3 cells (Figure 1A).

To confirm Nkx3.1-His expression in stably transfected PC3 cells, Western blotting was performed using the lysates. Figure 1B shows the results for anti-Nkx3.1 and anti-His. There was a basal level of Nkx3.1 in LNCaP cells (38 kDa). No detectable Nkx3.1 protein was seen in pcDNA3.1-PC3 cells or PC3 cells, whereas Nkx3.1-His delivery enabled a high expression of Nkx3.1 fusion protein (approximately 39 kDa). To further assess the Nkx3.1 fusion protein, Western blotting was performed using anti-His. The results indicate that the expression of His was in accordance with Nkx3.1.

3.2 Nkx3.1 enhances 17β-estradiol antiproliferative effect in PC3 cells

To assess the potential role of *Nkx3.1* in human prostate cancer PC3 cell growth, PC3 cells were stably transfected with Nkx3.1-pcDNA3.1 or pcDNA3.1. Figure 2A shows the effect of *Nkx3.1* on the proliferation of PC3 cells. Proliferation status was assessed by MTT assay. Our results demonstrate that stably transfected PC3 cells fail to produce a measurable difference between Nkx3.1-PC3 group and pcDNA3.1-PC3 group at any of the time point.

Next, we addressed the question of whether exogenous

Nkx3.1 expression could enhance the antiproliferative effect of 17β-estradiol in PC3 cells. Figure 2B shows the effect of 17β-estradiol on the growth of Nkx3.1-PC3 and pcDNA3.1-PC3 cells after 5 days of exposure. Nkx3.1-PC3 cells and pcDNA3.1-PC3 cells displayed a significant, dose-related inhibition of growth, with a maximal effect at 100 nmol/L of 17β- estradiol. Nkx3.1-PC3 cells produced a significantly lower absorbance at the 120 h time-point compared with the pcDNA3.1-PC3 cells at concentrations higher than 0.1 nmol/L 17β-estradiol (0.1 nmol/L, $P < 0.05$; 1 nmol/L, $P < 0.05$; 10 nmol/L, $P < 0.01$; 100 nmol/L, $P < 0.001$). The results revealed that stable expression of *Nkx3.1* in PC3 cells could enhance the antiproliferative effect of 17β-estradiol.

3.3 Nkx3.1 expression-promoted 17β-estradiol-induced apoptosis of PC3 cells

The significant inhibition of proliferative activity in PC3 human prostate cancer cells by 17β-estradiol has been proven. To clarify whether *Nkx3.1* expression-promoted 17β-estradiol -inhibited cell growth was a result of the induction of apoptosis, an alternative evaluation of apoptosis was completed by means of Annexin V-FITC analysis. With this assay, apoptotic and necrotic subpopulations could be distinguished. The apoptotic rates of Nkx3.1-PC3 and pcDNA3.1-PC3 cells were quantified by Annexin V and PI double staining followed by cytometry analysis after 5 days of 17β-estradiol exposure (Figure 3A and B). The loss of plasma membrane asymmetry is an early event in apoptosis and could result in the exposure of phosphatidylserine residues at the outer plasma membrane leaflet. Annexin V, a phospholipid binding protein, specifically binds to phosphatidylserine residues. The Annexin V-FITC binding assay

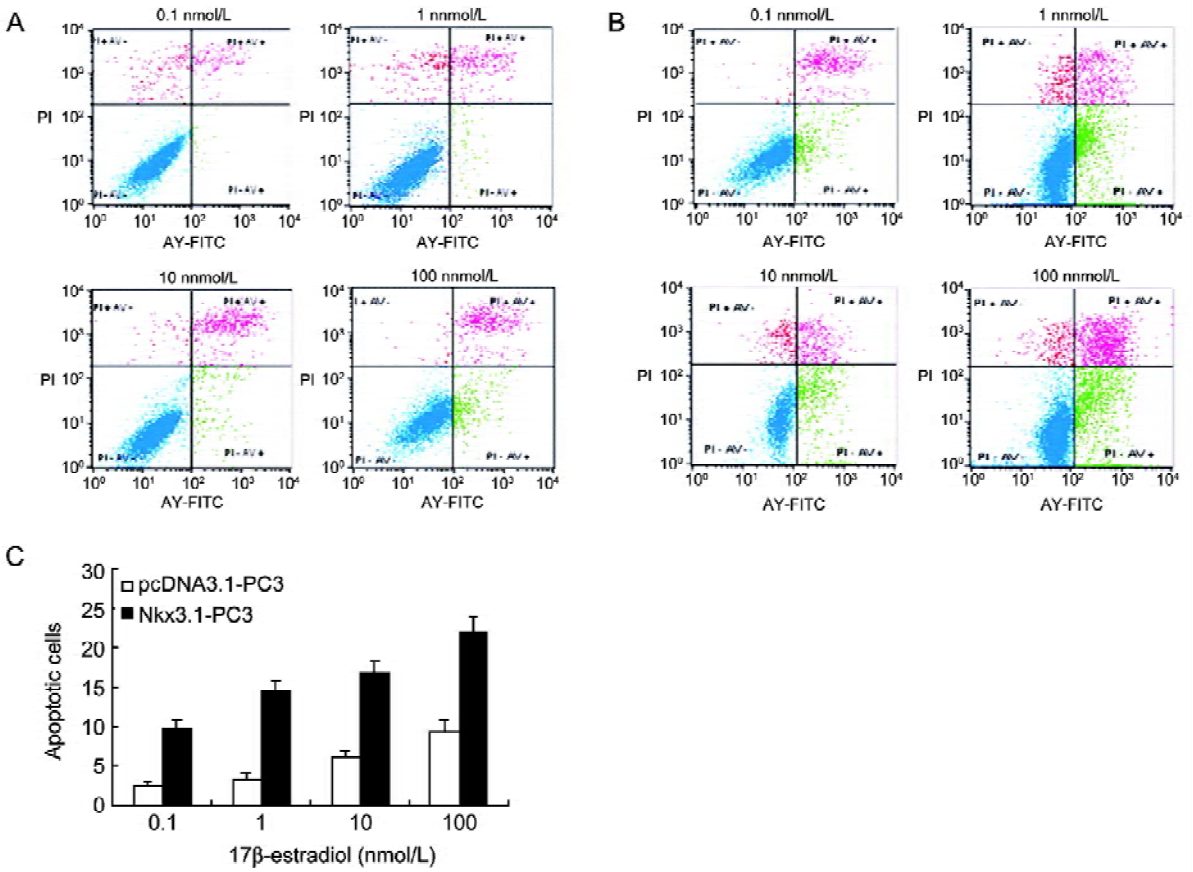

Figure 3. *Nkx3.1* expression promotes 17β-estradiol-induced apoptosis of PC3 cells. (A): The apoptotic of pcDNA3.1-PC3 cells with 17β-estradiol treatment. After 5 days of exposure, cells were quantified by Annexin V (AY-FITC) and PI double staining followed by cytometry analysis. (B): The apoptotic Nkx3.1-PC3 cells with 17β-estradiol treatment. (C): Quantitative analysis of apoptotic cells from A and B.

showed that pcDNA3.1-PC3 cells displayed a slight, dose-related increase of apoptosis, whereas a striking increase in apoptotic cells was observed in the Nkx3.1-PC3 group (Figure 3C). The results show that Nkx3.1 expression promoted 17β-estradiol-induced apoptosis of PC3 cells. However, these results also indicate that expression of *Nkx3.1* enhances the 17β-estradiol antiproliferative effect, at least in part as a result of the higher proportion of apoptotic cells in the Nkx3.1-PC3 group.

3.4 Expression of apoptosis-related protein in Nkx3.1-PC3 and pcDNA3.1-PC3 cells treated with 17β-estradiol

The effect of different treatments on expression of the Bcl-2 family of anti-apoptotic and pro-apoptotic proteins was determined to gain insights into the mechanism for apoptosis. The protein expression levels of Bcl-2 and Bax in Nkx3.1-PC3 and pcDNA3.1-PC3 cells treated with 0.1 nmol/L, 1 nnmol/L,10 nnmol/L or 100 nnmol/L of 17β-estradiol for 5 days was analyzed by Western blot analysis. As shown in Figure 4, there was striking downregulation of the level of Bcl-2 expression in Nkx3.1-PC3 cells treated with 1 nnmol/L, 10 nnmol/L or 100 nnmol/L of 17β-estradiol. In contrast, the expression level of Bax was upregulated. These observations indicate that *Nkx3.1* expression-promoted 17β-estradiol-induced apoptosis might be manifested by an increase in the ratio of Bax to Bcl-2.

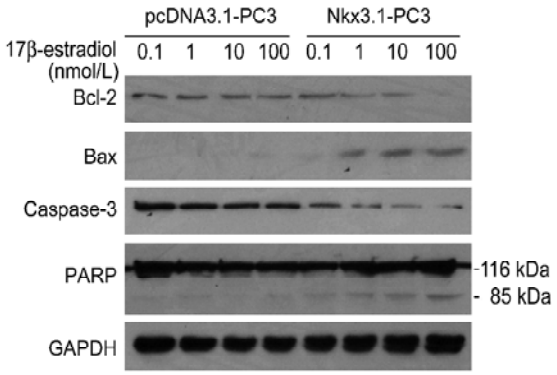

Figure 4. Western blot analysis of apoptosis-related proteins expressed in pcDNA3.1-PC3 cells and Nkx3.1-PC3 cells after treatment with 17β-estradiol. After 5 days of exposure, cell lysates were harvested and Bcl-2, Bax, Caspase-3 and poly(ADP-ribose) polymerase (PARP) were detected by Western blot. Glyceraldehydes-3-phosphate dehydrogease (GAPDH) was used as a loading control.

The effect of 17β-estradiol on Nkx3.1-PC3 cellular apoptosis corresponded to a decrease in caspase-3 protein expression (caspase-3 activation is presented by the loss of its pro-form). Caspase-3 is one of the executioner caspases in response to the activation of the intrinsic mitochondrial apoptotic pathway, which can be triggered by a blockade of ErbB signaling [11, 12]. When caspase-3 is actived, the intact form of the 33 kDa is cleaved into an activated form of 17/19 kDa, which in turn cleaves PARP [12]. Consistent with the caspase-3 activation results, 17β-estradiol markedly increased the level of cleaved PARP in Nkx3.1-PC3 cells. The results suggest that increased apoptosis induced by the 17β-estradiol in Nkx3.1-PC3 cells is triggered by activation of caspase-3 and regulated by Bcl-2 family proteins.

4 Discussion

Prostate cancer is one of the most common human malignancies: thus far, there has been no effective therapy for the treatment of AIPC. In the present study, we developed an *in vitro* system; namely, PC3 cell lines that were stably transfected with Nkx3.1 cDNA. Although Nkx3.1 failed to produce significant growth inhibition, we found that exogenous expression of Nkx3.1 protein in human prostate cancer PC3 cells promoted the antiproliferative effect of 17β-estradiol, which was associated with cellular apoptosis. The fact that *Nkx3.1* expression-promoted 17β-estradiol-induced apoptosis might be a result of an increase in ratio of Bax to Bcl-2 further indicates that caspase-3 is activated, which in turn cleaves PARP. These results suggest that the combining use of *Nkx3.1* and 17β-estradiol possesses novel anti-tumor action on PC3 human prostate cancer cells.

There are some divergent viewpoints about whether *Nkx3.1* is a true tumor suppressor gene. Bhatia-Gaur *et al.* [13] consider *Nkx3.1* to be a prostate-specific tumor suppressor gene and posit that loss of a single allele might predispose to prostate carcinogenesis. Kim *et al.* [14] found that overexpression of *Nkx3.1* resulted in an approximate 70% reduction in cellular proliferation in rodent AT6 cells and a 60% reduction in PC3 cells. Lei *et al.* [15] demonstrate that *Nkx3.1* inhibits AKT phosphorylation/activation through an AR-dependent mechanism and show that Nkx3.1 expression *in vivo* can block the hyperproliferative and antiapoptotic effects brought on by phosphatase and tensin homolog (PTEN, mutated in multiple advanced cancers 1) loss. In contrast, some

scholars suggest that *Nkx3.1* does not function as a typical tumor suppressor protein in prostate cancer, such as P53, retinoblastoma or PTEN [8, 9, 16]. Instead, *Nkx3.1* appears to act more like a tumor modulator, serving as a regulator of differentiation, which in turn prevents cancer initiation. In the present study, we found that overexpression of exogenous *Nkx3.1* did not display a significant reduction in cellular proliferation. Our results provide support for the idea that *Nkx3.1* does not function as a typical tumor suppressor gene: by restoring *Nkx3.1* expression it is not sufficient for inhibiting growth of PC3 cells.

Interest in estrogen therapy has also been rekindled by recent trials suggesting that parenteral administration of estrogens might result in the avoidance of much cardiovascular toxicity [4]. Most recently, Ockrim *et al.* [17] reported encouraging biochemical results in 20 patients with locally advanced or metastatic cancer treated with transdermal estradiol patches. Nevertheless, estrogens produced biochemical responses only in one-quarter to two-thirds of patients in whom primary androgen deprivation therapy failed [1, 18]. Therefore, enhancing estrogen anti-tumor action might have the most significant clinical impact. We found that 17β-estradiol is an effective inhibitor of human prostate cancer PC3 cells. Our result is in agreement with a report by Carruba *et al.* [19], which also stated that 17β-estradiol is an effective inhibitor of PC3 cells and displays a significant, dose-related inhibition of growth. In our study, to investigate the effects of the combination of *Nkx3.1* transfection and 17β-estradiol on PC3 cells growth *in vitro*, Nkx3.1-PC3 cells and pcDNA3.1-PC3 cells were incubated with increasing concentrations of 17β-estradiol, from 0.01 nmol/L up to 100 nmol/L. Our results demonstrate that plasmid stably transfected from *Nkx3.1* into PC3 cells that do not express this gene, resulted in a significantly higher antiproliferative effect of 17β-estradiol (0.1 nmol/L or more) against PC3 cells compared to the control group. Consistent with our proliferation assay, stable expression of *Nkx3.1* promotes 17β-estradiol-induced apoptosis. These results suggest that the effect of the Nkx3.1-enhanced 17β-estradiol antiproliferative effect is a result of the Nkx3.1-amplified 17β-estradiol-induced apoptosis.

In our study, we also tried to elucidate some molecular mechanisms by which Nkx3.1 expression-promoted 17β-estradiol-induced apoptosis of PC3 cells. Several pathways are known to lead to apoptosis. The Bcl-2 family and caspase-3 are important regulators of apoptosis. Several studies demonstrate that the increased expression of Bcl-2 in prostate cancer confers androgen resistance, particularly in advanced disease, and might facilitate progression to androgen independence [20, 21]. Bcl-2 is part of an expanding family of apoptosis-regulatory molecules, which might act as either death antagonists (Bcl-2, Bcl-xl and Mcl-1) or death agonists (Bax, Bak, Bcl-xS, Bad and Bid). The selective and competitive dimerization between pairs of antagonists and agonists determines how a cell will respond to a given signal. It has been reported that Bax inactivates Bcl-2 proteins, which protect cells from apoptosis and that the ratio of Bax/Bcl-2 increases during apoptosis [22, 23]. The Bax protein levels were increased in the 17β-estradiol-treated Nkx3.1-PC3 cells, leading to an increase in the ratio of Bax to Bcl-2, which might be responsible for inducing apoptotic processes in our system. However, the precise molecular actions on the members of the Bcl-2 family require further investigation. Nevertheless, it is clear that Nkx3.1 expression-promoted apoptosis by 17β-estradiol on PC3 cells is at least in part a result of inactivation of Bcl-2 and a great elevation of Bax expression.

In normal prostate cells, PTEN inhibits the phosphatidylinositol 3-kinase (PI3K) pathway. Activation of this pathway stimulates Akt, which inactivates several proapoptotic proteins, therefore enhancing cell survival. During androgen-independent progression, the loss of PTEN reverses the inhibition of the PI3K-Akt pathway, permitting activated Akt to phosphorylate Bad. This activation results in the release of Bcl-2, which eventually leads to cell survival [24]. It is of value to further address whether the PI3K-Akt signal pathway has been connected with the alteration of Bcl-2 and Bax proteins expression during Nkx3.1 expression-promoted 17β-estradiol-induced apoptosis.

Caspase activation leads to cleavage and inactivation of key cellular proteins, such as poly (ADP-ribose) polymerase [25]. We found that the activation of caspase-3 was enhanced by 17β-estradiol in Nkx3.1-PC3 cells. Consistent with the caspase-3 activation results, 17β-estradiol markedly increased the level of cleaved PARP in Nkx3.1-PC3 cells. Caspase-3 is an executioner caspase that can be activated by a mitochondrial pathway involving caspase-9 or a death receptor pathway involving caspase-8 [25]. Of particular interest in our finding is that Nkx3.1 expression-promoted 17β-estradiol-induced apoptosis appears to involve the mitochondrial pathways, as demonstrated by the increase in Bax protein expression and the cleaved PARP.

In summary, this report describes a potentially useful approach in the treatement of AIPC: the expression of *Nkx3.1* enhances 17β-estradiol anti-proliferative effects on PC3 cells. In particular, expression of *Nkx3.1* promotes 17β-estradiol multiple anti-tumor effects of activation of caspase-3 and PARP, downregulation of Bcl-2 protein and upregulation of Bax protein expression, triggering cellular apoptosis. Our *in vitro* study suggests that the re-expression of *Nkx3.1* is worthy of further consideration as an adjuvant method in the treatment of AIPC with estrogen anti-tumor therapy.

Acknowledgment

We would thank Prof. Rong-Zhen Xu for the expert technical assistance in the study.

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Edited by Drs Sidney Grimes and Robert H. Getzenberg