

# Estrogen in combination with 5-azacitidine up-regulates p75NTR expression and induces apoptosis in 22Rv1 prostate cancer cells

JIAN-DI YU<sup>1,2</sup>, KAI YANG<sup>1</sup>, QI-QI MAO<sup>1</sup>, DE-BO KONG<sup>1</sup>, XIANG-YI ZHENG<sup>1</sup> and LI-PING XIE<sup>1</sup>

<sup>1</sup>Department of Urology, First Affiliated Hospital, School of Medicine, Zhejiang University, Qingchun Road 79, Hangzhou 310003; <sup>2</sup>Department of Urology, Zhejiang Hospital, Lingyin Road 12, Hangzhou 310013, Zhejiang, P.R. China

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**Abstract.** Previous studies suggest that the low-affinity neurotrophin receptor p75NTR inhibits the proliferation of human prostate cancer cells, and that estrogen interacts with p75NTR in many tissues. In this study, we exposed 22Rv1 androgen-independent prostate cancer cells to 17- $\beta$ -estradiol and the DNA demethylating agent 5-azacitidine (5-AzaC) to explore the interactions between estrogen and p75NTR. We found that estrogen induced estrogen receptor (ESR) subtype 2 and p75NTR expression in 22Rv1 cells, and that 5-AzaC further enhanced these effects. Estrogen in combination with 5-AzaC induced cell apoptosis, which was associated with the inhibition of NF- $\kappa$ B translocation to the nucleus. These results provide evidence that the up-regulation of ESR2 and p75NTR by estrogen plus 5-AzaC may be a potential therapeutic strategy for the treatment of androgen-refractory prostate cancer.

## Introduction

Prostate cancer is the most common male malignancy in western countries, and usually manifests initially as an androgen-dependent disease (1). Although localized disease can be cured by surgery and radiation therapy, the most commonly applied therapy for advanced disease is androgen ablation. Androgen-ablation therapy almost always generates effective clinical responses initially; however, most patients inevitably relapse with androgen-refractory prostate cancer, which ultimately leads to death. Peptide growth factors play a major role in the control of normal and malignant growth of the prostate, and

probably contribute to the development of androgen-refractory prostate cancer during androgen-ablation therapy (2).

Estrogen is commonly used for the treatment of prostate cancer by androgen ablation, and is thought to act by blocking the hypothalamic-pituitary-testicular axis (3). However, recent studies have demonstrated that estrogen can exert direct growth-inhibitory effects on androgen-refractory prostatic cancer cells via the induction of mitotic arrest or apoptosis (4,5). The biological effects of estrogen are mainly thought to be mediated by two estrogen receptor (ESR) subtypes, ESR1 and 2, which are ligand-dependent transcription factors belonging to the steroid/thyroid nuclear receptor superfamily (6). ESR2, the predominant ESR subtype expressed in the prostate, is linked to increased cell differentiation and the inhibition of cell proliferation in normal and neoplastic epithelial cells (7). In a previous study, the expression of ESR2 was suppressed in prostate cancer cell lines, which was believed to be associated with epigenetic silencing by CpG island methylation of the gene promoter (8). Treatment with the DNA demethylating agent 5-azacitidine (5-AzaC) can reactivate ESR2, inducing apoptosis in prostate cancer cell lines (9).

Studies have revealed a prominent role for neurotrophins in the regulation of prostate growth. It has been reported that neurotrophins stimulate cell proliferation in androgen-refractory prostate cancer cell lines (10), exerting their effects by binding to the corresponding receptors (11). There are two types of neurotrophin receptors present in the prostate, the proto-oncogene tyrosine receptor kinase trkA and the 75-kDa glycoprotein p75NTR, which is a member of the TNF receptor superfamily. p75NTR has been proposed to be a tumor suppressor gene for the prostate, and is progressively lost during tumorigenesis (12). Inducing or re-expressing p75NTR in prostate cancer cells can promote apoptosis via p75NTR-dependent inhibition of the translocation of NF- $\kappa$ B and phosphorylated JNK to the nucleus (13).

Estrogen and its receptors can interact with neurotrophins and their receptors. Estrogen receptors are co-localized in cells that express neurotrophins and their receptors, and estrogen further regulates the expression of the neurotrophic system in many tissues, not just the nervous system (14,15). Estrogen treatment can induce the expression of p75NTR in

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*Correspondence to:* Dr Li-Ping Xie, Department of Urology, First Affiliated Hospital, School of Medicine, Zhejiang University, Qingchun Road 79, Hangzhou 310003, Zhejiang, P.R. China  
E-mail: lpxie@yahoo.cn

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uterine cells and in cholinergic neurons (16,17), but little is known regarding its effects in the prostate. We speculated that interactions between estrogen and the neurotrophic system may partially explain the beneficial effects of estrogen therapy for androgen-refractory prostate cancer.

In this study, we examined the effects of 17- $\beta$ -estradiol and 5-AzaC administration on the expression of ESRs, the neurotrophin receptor TrkA and p75NTR in the 22Rv1 androgen-independent prostate cancer cell line, which exhibits low expression levels of ESRs and p75NTR (18,19). We found that estrogen induced the expression of p75NTR and ESR2 in the 22Rv1 cells. 5-AzaC further enhanced this effect by stimulating the expression of ESR2. Combined treatment induced the apoptosis of the prostate cancer cells, which was related to the inhibition of NF- $\kappa$ B translocation to the nucleus.

## Materials and methods

**Reagents.** 17- $\beta$ -estradiol and 5-AzaC were purchased from Sigma-Aldrich (Milan, Italy). The primary immunoblotting antibodies were anti-ESR1, anti-ESR2, anti-TrkA, anti-p75NTR, anti- $\beta$ -actin (Cell Signaling Technology, Beverly, MA, USA) and anti-NF- $\kappa$ B (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA).

**Cell culture and treatment.** The human prostate cancer cell line 22Rv1 was obtained from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences. The cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 mg/l) in a humidified atmosphere containing 5% CO<sub>2</sub> maintained at 37°C. 17- $\beta$ -estradiol was dissolved in ethanol, and 5-AzaC was dissolved in RPMI-1640 medium; the maximum ethanol concentration per Petri dish was <0.1%. The day before treatment, cells were plated in growth medium without antibiotics at different densities so that all time-course studies were carried out on exponentially growing cells. After overnight incubation, cells were treated for the appropriate times with a single dose of 10<sup>-6</sup> M 17- $\beta$ -estradiol and/or 2  $\mu$ g/ml 5-AzaC, administered every 24 h according to previous studies, which showed that this dose was associated with gene re-expression without direct toxicity (7,8). The treatment period lasted for 72 h, and cell images were captured every 24 h using a phase-contrast microscope at x100 magnification (Olympus, Japan).

**Cell growth/cell viability assay.** Cell proliferation was determined by the MTT assay. Approximately 3000-8000 (depending on how long the cells were to be cultured) 22Rv1 cells were plated in each well of a 96-well plate. After overnight incubation, the cells were treated with 17- $\beta$ -estradiol and 5-AzaC, alone or in combination, for 48 h. The concentration of 17- $\beta$ -estradiol ranged from 10<sup>-9</sup> to 10<sup>-5</sup> M, and the final concentration of 5-AzaC was 2  $\mu$ g/ml in each well. At 48 h after treatment, MTT (20  $\mu$ l of 5 mg/ml) was added to each well and incubated at 37°C for 4 h. Then, the plates were spun, and the purple-colored formazan precipitates were dissolved in 150  $\mu$ l dimethylsulfoxide. Absorbance was measured at 490 nm using the MRX II absorbance reader (Dynex Technologies, Chantilly, VA, USA). The reduction in viability of the treated 22Rv1

cells was expressed as a percentage compared with the control cells. The control cells were considered to be 100% viable.

**Analysis of cell apoptosis by flow cytometry.** Quantitative assessment of apoptosis was conducted by determining the percentage of cells with nuclei that were highly condensed or fragmented. Cells were harvested at 72 h after treatment as described above, washed twice with pre-chilled PBS and resuspended in 100  $\mu$ l of 1X binding buffer at a concentration of 10<sup>6</sup> cells/ml. Annexin V and PI double-staining was performed using the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA, USA) in accordance with the manufacturer's instructions. Analysis of cell apoptosis was performed using a Beckman Coulter FC500 Flow Cytometry System with CXP Software (Beckman Coulter, Fullerton, CA, USA) within 1 h.

**Semi-quantitative RT-PCR.** Total cellular RNA was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) in accordance with the manufacturer's instructions. RNA (3  $\mu$ g) was reverse transcribed using oligo-dT primers and M-MLV Reverse Transcriptase (Promega, Madison, WI, USA). Following reverse transcription, cDNA was amplified by PCR using primers specific for each gene. The PCR primers were 5'-AAAGGTGGGATACGAAA-3' (sense) and 5'-CAA GAGCAAGTTAGGAGC-3' (antisense) for ESR1, 5'-GCT TTGGTTTGGGTGATT-3' (sense) and 5'-CCGAGTTGATTA GAGGGTC-3' (antisense) for ESR2, 5'-TGAGTGCTGCAA AGCCTGCAA-3' (sense) and 5'-TCTCATCCTGGTAGTAGC CGT-3' (antisense) for p75NTR, 5'-CCATCGTGAAGAGTG GTCTC-3' (sense) and 5'-GGTGACATTGGCCAGGGTCA-3' (antisense) for trkA, and 5'-TGATGACATCAAGAAGGTGGT GAAG-3' (sense) and 5'-TCCTTGGAGGCCATGTGGGCC AT-3' (anti-sense) for GAPDH. The PCR products were separated by electrophoresis through a 1.5% agarose gel containing ethidium bromide, and the bands were visualized by ultraviolet light.

**Western blot analysis.** Briefly, cells were harvested at 72 h after  $\beta$ -estradiol and/or 5-AzaC treatment as described above, and washed and lysed with lysis buffer. The protein concentration in the resulting lysate was determined using a bicinchoninic acid protein assay kit (Pierce Biotechnology, Rockford, IL). Appropriate amounts of protein (30-50  $\mu$ g) were resolved by electrophoresis in 10-15% Tris-glycine polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked and then incubated overnight with the appropriate primary antibody at dilutions specified by the manufacturer. The membranes were then washed three times in 15 ml Tris-buffer saline Tween-20 (TBST) and incubated with the corresponding horseradish peroxidase-conjugated secondary antibody at 1:1000 dilution in TBST for 1 h. After washing three times for 5 min each with 15 ml TBST, the bound secondary antibody was detected using an enhanced chemiluminescence (ECL) system (Pierce Biotechnology Inc.).

**Statistical analysis.** All values were expressed as the means  $\pm$  SD. Statistical significance was compared using two-way ANOVA by SPSS statistical software (version 11.5, SPSS Inc.).  $p < 0.05$  was considered statistically significant.

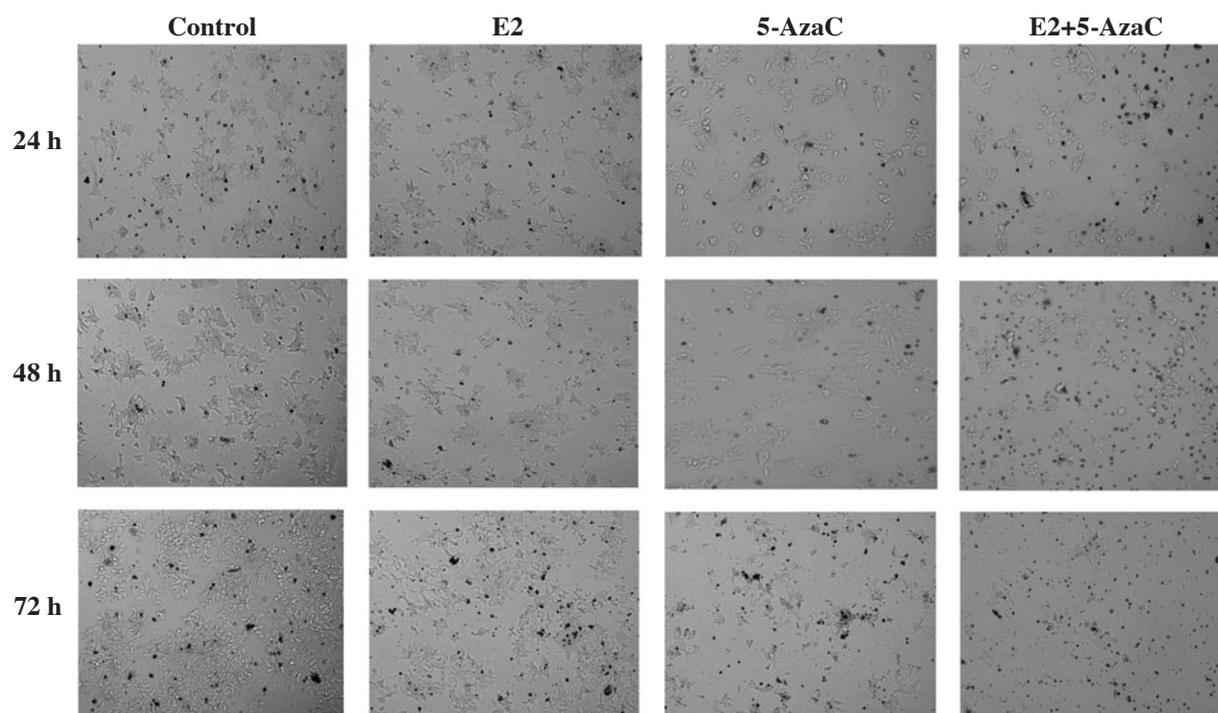


Figure 1. 17- $\beta$ -estradiol- and/or 5-AzaC-inhibited 22Rv1 cell growth. 22Rv1 cells were treated with  $10^{-6}$  M 17- $\beta$ -estradiol and/or 2  $\mu$ g/ml 5-AzaC. Cell images were captured at 24, 48 and 72 h after treatment (x100 magnification). The 22Rv1 cells treated with 17- $\beta$ -estradiol and 5-AzaC were less dense, and there were more dead cells in this group compared with the groups of cells treated with individual agents or the control.

## Results

**Estrogen and 5-AzaC inhibit 22Rv1 cell proliferation.** By visual inspection of cells through a phase-contrast microscope, we found that the administration of 17- $\beta$ -estradiol or 5-AzaC alone inhibited the proliferation of 22Rv1 cells. A more pronounced effect was observed after co-administration of the two agents, as shown in Fig. 1. Moreover, in addition to reduced cell numbers, the cells changed morphologically. Typical changes included cell membrane shrinkage and membrane blebbing, which are consistent with apoptosis.

The effects of varying concentrations of 17- $\beta$ -estradiol alone and of 17- $\beta$ -estradiol in combination with 5-AzaC on cell proliferation were subsequently determined using the MTT assay. 5-AzaC was also administered alone to determine its effects. As shown in Fig. 2A, 17- $\beta$ -estradiol inhibited the proliferation of 22Rv1 cells in a dose-dependent manner. The optimal concentration of 17- $\beta$ -estradiol was  $10^{-6}$  M, which reduced cell viability by  $\sim 13.87\%$  after 48 h. The effects were further enhanced by co-administration of 5-AzaC (2  $\mu$ g/ml); at the same concentration of 17- $\beta$ -estradiol, cell viability was decreased by 41.8% after 48 h. Treatment with 5-AzaC (2  $\mu$ g/ml) alone reduced cell viability by 15.34% after 48 h. The effect of 5-AzaC was slightly more potent than that of 17- $\beta$ -estradiol. The reduction in cell viability by co-administration of the agents was significantly greater than that achieved by administration of a single agent ( $p < 0.05$ ), indicating an additive effect of co-administration.

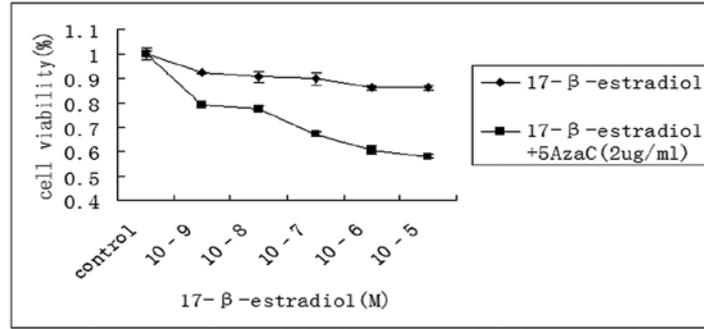
**Estrogen and 5-AzaC induce cell apoptosis.** Since the MTT assay revealed that 17- $\beta$ -estradiol and 5-AzaC co-administration

inhibits the growth of 22Rv1 prostate cancer cells, flow cytometry was used to identify whether this growth inhibition was related to apoptosis. As shown in Fig. 2B, there was marked apoptosis in the 22Rv1 prostate cancer cells at 72 h after 17- $\beta$ -estradiol and 5-AzaC administration. As compared to the control group, co-administration induced a 12-fold increase in the number of apoptotic 22Rv1 cells. The analysis revealed that 52.9% of the cancer cells underwent apoptosis within 72 h after co-treatment, whereas administration of a single agent (estrogen or 5-AzaC) induced less cell apoptosis; 23.6% for estrogen and 29.2% for 5-AzaC. In contrast, cells in the control group demonstrated normal cell viability without marked cell apoptosis or death.

**Estrogen and 5-AzaC up-regulate ESR2 and p75NTR in 22Rv1 cells.** The expression of ESRs, trkA and p75NTR in 22Rv1 prostate cancer cells was determined using RT-PCR and Western blotting. As shown in Fig. 3, the administration of 17- $\beta$ -estradiol or 5-AzaC alone induced the mRNA and protein expression of ESR2 in 22Rv1 prostate cancer cells; however, the most significant effects were observed in the co-treated group. The mRNA level of ESR2 induced by 5-AzaC or by co-administration was higher than that induced by estrogen alone. Similar results were observed for ESR2 protein expression. However, both 17- $\beta$ -estradiol and 5-AzaC failed to induce ESR1 expression in this cell line.

In contrast, treating 22Rv1 prostate cancer cell with 17- $\beta$ -estradiol alone enhanced the protein expression of p75NTR 2-fold compared with the controls, while treatment with 5-AzaC alone only slightly enhanced the protein level of p75NTR (Fig. 3C). Consistent with ESR2, the expression of

A.



B.

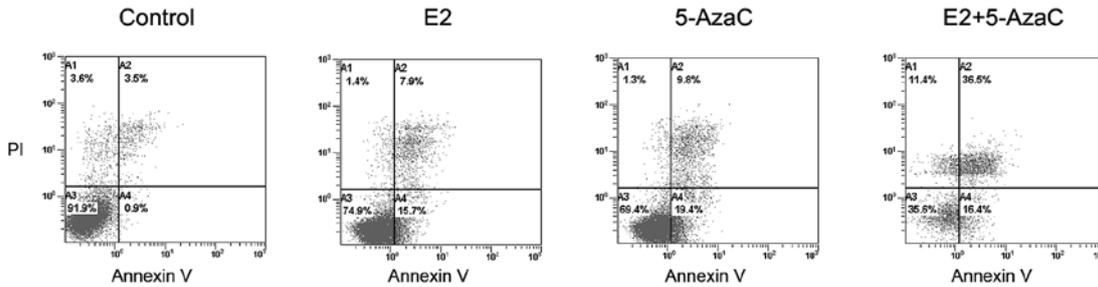
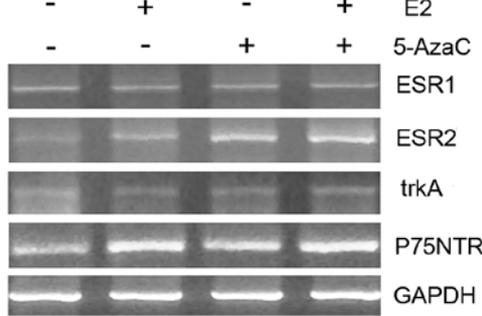
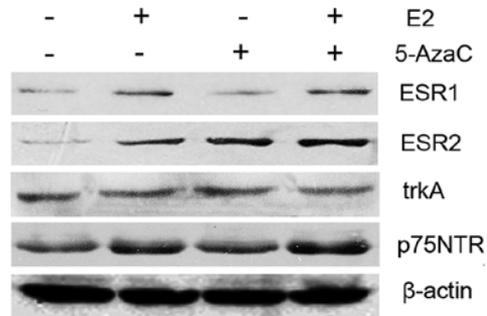


Figure 2. (A) Dose-dependent inhibition of cell viability of human prostate cancer 22Rv1 cells by 17-β-estradiol alone or in combination with 5-AzaC, assessed using the MTT assay. Reduced cell viability was observed with 17-β-estradiol alone (10<sup>-9</sup> to 10<sup>-5</sup> M) or in combination with 5-AzaC (2 μg/ml) at 48 h. Data are presented as the means ± SD (n=6). (B) 17-β-estradiol- and/or 5-AzaC-induced apoptosis in 22Rv1 human prostate cancer cells, detected by flow cytometry using a double-staining method with FITC-conjugated Annexin V and PI. Annexin V-stained cells are early apoptotic, whereas cells stained with Annexin V and propidium iodide are late apoptotic. Results are presented as the means ± SD of three independent experiments.

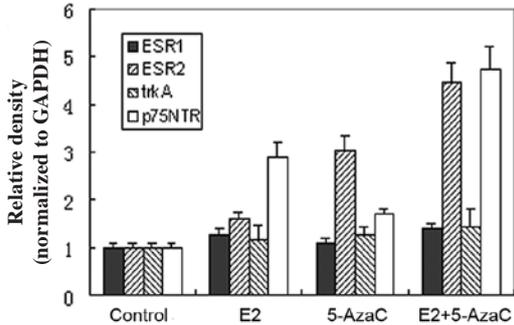
A.



C.



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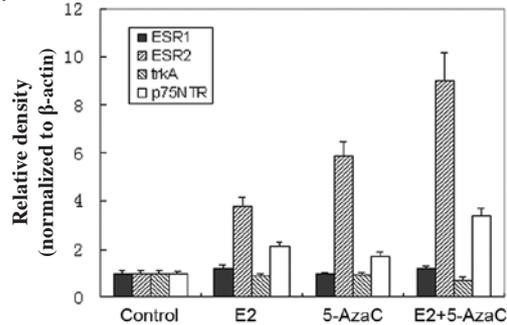


Figure 3. Expression of ESRs, trkA and p75NTR mRNA and proteins in cells treated with 17-β-estradiol and/or 5-AzaC, analyzed by RT-PCR and Western blotting (see Materials and methods). GAPDH levels in RT-PCR and β-actin levels in Western blotting were also detected and served as a loading control. A representative blot from three independent experiments with identical results is shown. (A) mRNA expression of ESRs, trkA and p75NTR in 22Rv1 human prostate cancer cells at 72 h after 17-β-estradiol and/or 5-AzaC treatment. (B) mRNA expression of ESRs and trkA and p75NTR normalized to GAPDH. Results are presented as the means ± SD of three independent experiments. (C) Protein expression of ESRs, trkA and p75NTR in 22Rv1 human prostate cancer cells at 72 h after treatment with 17-β-estradiol (10<sup>-6</sup> M) and/or 5-AzaC (2 μg/ml). (D) Protein expression of ESRs, trkA and p75NTR normalized to β-actin expression. Results are presented as the means ± SD of three independent experiments.

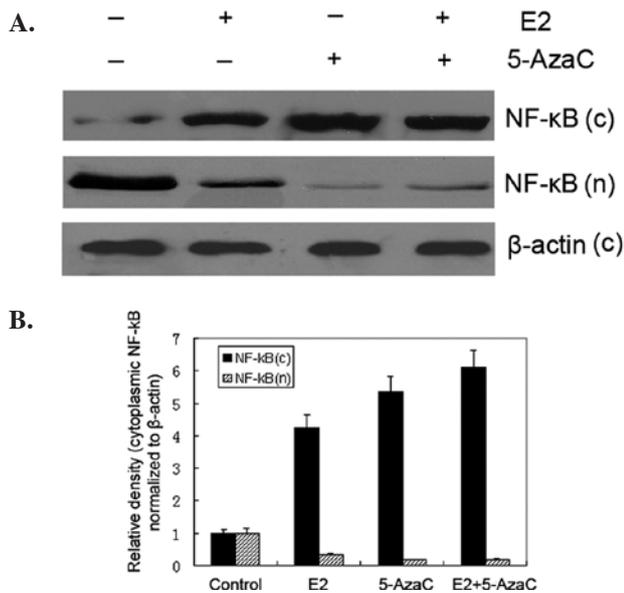


Figure 4. 17- $\beta$ -estradiol ( $10^{-6}$  M) and/or 5-AzaC ( $2 \mu\text{g/ml}$ ) treatment inhibits the translocation of NF- $\kappa$ B from the cytoplasm to the nucleus. (A) Relatively less NF- $\kappa$ B was detected in the nucleus of cells treated with 17- $\beta$ -estradiol ( $10^{-6}$  M) in combination with 5-AzaC ( $2 \mu\text{g/ml}$ ) compared with estrogen treatment alone or with the control groups. c, cytoplasm; n, nucleus. (B) Protein expression of cytoplasmic and nuclear NF- $\kappa$ B was normalized to that of cytoplasmic  $\beta$ -actin. Results are presented as the means  $\pm$  SD of three independent experiments.

p75NTR was more markedly enhanced by co-treatment with 17- $\beta$ -estradiol and 5-AzaC than by any single agent. mRNA and protein expression of trkA were not affected by either treatment compared with the control group.

**Estrogen and 5-AzaC inhibit the translocation of NF- $\kappa$ B to the nucleus.** Previous studies suggest that ESR2 or p75NTR induces apoptosis in prostate cancer cells by inhibiting the NF- $\kappa$ B signaling pathway (13,20). In this study, we used Western blotting to detect the regulation of NF- $\kappa$ B by estrogen and 5-AzaC. We found that 17- $\beta$ -estradiol and 5-AzaC, alone or in combination, inhibited NF- $\kappa$ B translocation from the cytoplasm to the nucleus (Fig. 4). Consistent with previous results, the effects of combined treatment on the inhibition of NF- $\kappa$ B were more pronounced than treatment with 17- $\beta$ -estradiol alone.

## Discussion

Estrogen has been used for the treatment of prostate cancer for several decades, and its direct and indirect effects on the inhibition of prostate cancer cell proliferation are well known (3-6,21). However, clinical use has been restricted owing to its adverse side effects, which mainly involve increased heart-vascular complications. Recently, interest in the use of estrogenic therapies for advanced prostate cancer has reemerged, since lower doses of estrogen have proven effective in subpopulations of patients with advanced prostate cancer (5,22,23). Additionally, the administration of estrogens parenterally, which avoids hepatic first-pass metabolism, appears to lower the risk of thromboembolism (24,25).

Estrogen requires its receptors to mediate biological activity. ESR2 is the main estrogen receptor in human prostate epithelial cells, and its expression is suppressed in prostate

cancer (26,27). The ESR2 gene contains a CpG island within its proximal promoter that is methylated in prostate cancer cell lines and in 79-100% of human prostate tumors. Treatment of prostate cancer cell lines with 5-AzaC can restore ESR2 expression (27,28). In our study, treatment with estrogen inhibited the proliferation of 22Rv1 prostate cancer cells and up-regulated ESR2 expression. Moreover, when 5-AzaC was added to reactivate the expression of ESR2, the effects of estrogen were markedly enhanced. However, the change in ESR1 expression failed to achieve statistical significance in all treated groups, indicating that ESR1 is not the predominant estrogen receptor subtype in these cells (7).

The low-affinity neurotrophin receptor p75NTR is a member of the TNF receptor superfamily and is widely expressed in the nervous system and in other tissues, including the prostate. p75NTR has been implicated in promoting cell apoptosis and death through a conserved intracellular death domain (29). p75NTR exhibits a partial loss of expression during malignant transformation of the prostate and is almost totally lost in most prostate cancer cell lines (30,31). However, the p75NTR gene remains intact in these cells, indicating that the potential for up-regulation still exists (11,32). Inducing or re-expressing this receptor in prostate cancer cells can induce cell cycle arrest and may facilitate apoptosis (33,34).

A number of studies suggest that estrogen can alter the expression of neurotrophin receptors and perhaps even neurotrophins (16,17,35). We first demonstrated that estrogen enhances the expression of p75NTR in 22Rv1 prostate cancer cells. This effect occurs in association with the up-regulated expression of ESR2, suggesting that estrogen might not only directly regulate p75NTR expression, but may also influence the neurotrophin pathways via their receptors. We also observed that p75NTR expression increased along with ESR2 in 22Rv1 cells treated with 5-AzaC alone, which indicates that p75NTR might be silenced by DNA promoter methylation. The exact mechanism of this needs to be further investigated in future studies.

A previous study revealed that NF- $\kappa$ B interacts with other pathways such as the nerve growth factor pathway, which is involved in the survival and proliferation of prostate cancer cells (36). Aside from this, recent studies have shown that both ESR2 and p75NTR induce apoptosis by inhibiting the translocation of NF- $\kappa$ B to the nucleus (13,20). We observed that the translocation of NF- $\kappa$ B was inhibited in cells exposed to estrogen or 5-AzaC alone or in combination, which was consistent with cell morphological changes, the results of flow cytometry and the up-regulation of ESR2 and p75NTR after treatment. These findings suggest that the apoptosis of 22Rv1 prostate cancer cells induced by estrogen and 5-AzaC might be partially associated with the inhibition of the NF- $\kappa$ B signaling pathway.

Taken together, our results demonstrate that estrogen directly inhibits proliferation and induces apoptosis in 22Rv1 androgen-independent prostate cancer cells. More pronounced effects are observed after the co-administration of estrogen with 5-AzaC, and may be mediated by the up-regulation of the expression of ESR2 and p75NTR, which cooperatively inhibits the NF- $\kappa$ B pathway. These results suggest that the up-regulation of ESR2 and p75NTR by estrogen in combination with 5-AzaC offers a potential therapeutic strategy for the treatment of androgen-independent prostate cancer.

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