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Original Article

Effect of ERβ-regulated ERK1/2 signaling on biological behaviors of prostate cancer cells

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Abstract: Estrogen receptor beta (ERβ) plays a role in prostate carcinogenesis. In this study, we investigated the effects of ERβ gene silencing in PC3 androgen-independent prostate cancer cells. PC3 cells were transfected with vector alone, scrambled shRNA vector, vector encoding ERβ-targeting shRNA (shERβ), or shERβ followed by addition of PD98059, a mitogen-activated protein kinase kinase (MEK) inhibitor (shERβ+PD98059). Cyclin D1, Bcl-2, matrix metalloproteinase (MMP)2, and phosphorylated (p-) extracellular signal-regulated kinase (ERK1/2) expression was detected by western blotting. While ERK1/2 expression was comparable in all cells, p-ERK1/2 expression was highest in shERβ cells, and lowest in shERβ+PD98059 cells. Bcl-2, cyclin D1, and MMP2 expression was highest and lowest in shERβ and shERβ+PD98059 cells, respectively. Flow cytometry analysis showed that ERβ silencing promoted cell proliferation by decreasing the percentage of cells in G0/G1. Analysis of colony formation, migration, and invasion capacities, measured using soft agar colony-formation, wound-healing, and transwell invasion assays, respectively, showed that ERβ silencing augments cell proliferation, migration, and invasion, and that this increase is reversed by PD98059 treatment. A tumor xenograft model in nude mice was used to assess the effect of ERβ silencing on the biological behavior of PC3 cells. Colony formation assays and tumor transplantation data indicated that ERβ silencing promotes tumor formation. Immunohistochemical analysis of tumors showed that vascular endothelial growth factor (VEGF) and p-ERK1/2 expression, but not that of total ERK1/2, was increased upon ERβ silencing. In conclusion, out data demonstrate that ERβ gene silencing enhances malignant biological behaviors of PC3 cells by activating the ERK1/2 signaling pathway.

Keywords: Estrogen receptor beta, ERK1/2, proliferation, apoptosis, prostate cancer

Introduction

Prostate cancer is the second most malignant tumor in men around the world [1]. The incidence of prostate cancer is increasing significantly in China [2]. Androgen therapy is the main regimen of treatment for metastatic prostate cancer. However, most patients eventually develop androgen-independent prostate cancer; creating an urgent need to further developing estrogen as an alternative therapeutic agent for androgen-independent prostate cancer [3, 4]. Estrogens can inhibit cell proliferation, as demonstrated in several experimental models [3, 5]. There is growing evidence that estrogen receptor beta (ERβ) protects against uncontrolled human breast cancer cell proliferation [6, 7]. Although activation of this receptor subtype is linked to increased cell differentiation and inhibition of cell proliferation [8], the impact of ERβ in prostate cancer cells is unclear.

Mitogen-activated protein kinases enhance cell survival by activating multiple signal transduction pathways. The extracellular signal-regulated kinase (ERK)1/2 signaling pathway, one of the classical Ras-Raf-MEK-ERK signal transduction pathways, plays an important role in the regulation of cell division, migration, and tumor invasion [9-11]. In many malignant tumors, such as in lung, breast, and ovarian cancers, the activation of ERK1/2 is widespread [12-14]. Some studies have showed that the occurrence of prostate cancer is closely related to the activation of the ERK1/2 pathway, and conversely, that the activation of the

ERK1/2 pathway engenders androgen-independent characteristics in prostate cancer cells [15]. ERβ may have a positive role in the inhibition of cell proliferation and the low ERβ expression in prostate cancer may be one mechanism through which prostate epithelial cells escape normal regulation in prostate cancer. However, the molecular mechanism through which ERβ regulates ERK1/2 signaling has not been elucidated yet.

In this study, we sought to examine the molecular mechanism(s) invoked by ERβ in prostate tumorigenesis. We evaluated the effect of ERβ gene silencing on cell proliferation and tumor formation/progression. In addition, the effect of ERβ on the ERK1/2 signaling pathway was also addressed using PC3 prostate cancer cells in which ERβ expression was silenced using short hairpin RNA (shRNA)-mediated methods, and a tumor xenograft mouse model.

Materials and methods

Cell culture and transfection

Human prostate carcinoma PC3 cells were obtained from the American Type Culture Collection and routinely cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS). Cells were plated on six-well plates and transfected (at 70-90% confluence) using 8 μL Lipofectamine 2000 (Invitrogen, CA) according to the manufacturer's instructions. The study included four treatment groups: blank control (BC), negative control (NC, transfection with pSilencer 2.1-U6-neo), shERβ (pSilencer 2.1-U6-neo-shERβ) and shERβ+PD98-059 (incubated with the MEK inhibitor PD98-059 after transfection of p Silencer 2.1-U6-neo-shERβ). Cells were harvested 48 h post-transfection for further analyses.

Plasmid construction

Plasmids containing the ERβ shRNA target sequence (5'-GTGTGAAGCAAGATCGCTA-3') were constructed by cloning the synthesized oligonucleotide into pSilencer 2.1-U6-neo vector (Ambion, Austin, TX) and used for gene silencing. The control pSilencer 2.1-U6-neo vector contained a scrambled sequence 5'-TCTTAATCGCGTATAAGGC-3'. All constructs were confirmed by DNA sequencing.

Real-time quantitative RT-PCR

Total RNA was isolated from PC3 cells using by Trizol Reagent (Invitrogen) according to standard procedures. For removal of genomic DNA, total RNA was incubated with DNase I. Then, MMLV reverse transcriptase (Promega) was used for cDNA synthesis according to the manufacturer's direction.

Real-time quantitative RT-PCR was carried out on the iCycler System (Bio-Rad). Comparative quantification was used, normalizing ERβ expression to an internal standard gene (β-actin). The followed primers were used: human β-actin, 5-CTGCGTCTGGACCTGGATGG-3 (forward) and 5'-CGATGGTGATGACCTGGCTGT-3' (reverse); human ERβ, 5'-AGAGTCCCTGGTGTGAAGCAA-3' (forward) and 5'-GACAGCGCAGAA GTGAGCATC-3' (reverse).

Antibodies used for western blotting

Western blotting was performed according to the method described in a previous report [16]. After transfer of resolved proteins, membranes were incubated with anti-ERK1/2 (ab31242; Abcam), anti-p-ERK1/2 (ab4822; Abcam), anti-ERβ (ab3576; Abcam), anti-VEGF (ab46154; Abcam), and anti-Bcl-2 (ab18210; Abcam) antibodies. After incubation with anti-MMP2 monoclonal (ab51125; Abcam) or anti-cyclin D1 monoclonal (ab16663; Abcam) antibodies, incubation with the secondary antibody (#7074; Cell Signaling) was carried out. Anti-β-actin monoclonal antibody (A3854; Sigma-Aldrich) was used to detect β-actin, the internal standard.

Flow cytometry analysis

For apoptosis measurements, the percentages of apoptotic cells were determined by flow cytometry using the Annexin V-FITC/PI cell apoptosis detection kit (Promega) according to the manufacturer's instructions. Cell proliferation was measured using the propidium iodide staining method [17].

Colony formation assay

Soft agar plates were prepared as described previously [18]. PC3 cells were transfected with either the pSilencer 2.1-U6-neo vector or shERβ plasmids for 48 h and incubated for two weeks

at 37°C in a 5% CO_2 incubator. The numbers of colonies larger than 2 mm were counted under a light microscope. Untransfected PC3 cells were used as the blank control.

Wound-healing assay

After transfection with the different plasmids for 24 h, cells were seeded in a 24-well plate and grown to confluence. The confluent monolayer of cells was wounded using a standard 200 μL pipette tip and then washed three times to remove the non-adherent cells. Wounds were monitored and photographed at the time the scrape was created and 24 h later. Cell migration capacity was calculated according to the width of the wounds at 0 h and 24 h. The migration rate is described as a percentage of the migration observed in the control group.

Matrigel invasion assay

The Matrigel invasion assay was conducted in 24-well plates and 8-μm Matrigel (Corning Inc., Corning). The membrane was coated with Matrigel. Twenty-four hours after transfection, cells were seeded into the upper compartment of the chamber in serum-free RPMI-1640 medium. The lower compartments of the chambers were filled with medium containing 20% FBS. Cells were cultured at 37°C and 5% CO_2 for 24 h, and then cells that had not invaded were removed by scraping off the top layer of the chamber. The invading cells were fixed with 100% methanol and stained with Gimesa and May-Grunwald solutions. Cells that had invaded the lower chamber were counted in five randomly selected fields and photographed using a microscope (Olympus CH-40; Olympus) at 200× magnification. All experiments were performed in triplicate.

Tumor xenograft assay

Tumor xenograft assay was carried out as described in our previous study [19]. PC3 cells were transfected with either pSilencer 2.1-U6-neo or shERβ plasmids, and the stably-transfected cell lines were cultured in the presence of 200 mg/mL G418 after transfection. For in vivo experiments, the flanks of three 4-to-5-week-old female BALB/c nude mice (Experimental Animal Center of the Hunan province) were injected with G418-resistant stably-transfected cells (6×10^7 cells per mouse). The mice were weighed at regular intervals (once every three days). After 22 days, the mice were sacrificed and the tumors were removed. The tumor volume was calculated. The tumor tissues were harvested and evaluated by immunohistochemical staining. The care and treatment of experimental animals were in accordance with institutional guidelines.

Statistical analysis

Data are presented as mean ± standard deviation. SPSS version 17.0 (Chicago, IL, USA) was used to analyze data. ANOVA and t-test were used to compare between-group differences. *P*-values less than 0.05 were considered statistically significant.

Results

Reduced expression of ERβ activates the p-ERK1/2 signaling pathway

The Ras/Raf/MEK/ERK signaling pathway mediates transduction of signals from the cell surface to transcription factors, ultimately resulting in alterations in gene expression. Activation of ERK, via MEK-mediated phosphorylation, induces phosphorylation of ERK targets and expression of cyclin D1, a key cell cycle regulator [20]. The Ras/Raf/MEK/ERK pathway also regulates apoptosis, in part by regulating phosphorylation of apoptosis-regulating proteins, including Bcl-2. ERK has also been shown to play a role in mediating tumor invasiveness, in part by regulating MMP-mediated breakdown of the extracellular matrix [21, 22]. Given the key roles of ERK targets, including cyclin D1, Bcl-2, and MMP-2, of the ERK signaling cascade in regulating cell cycle progression, apoptosis, and tumor invasiveness, we evaluated the impact of ERβ signaling on these ERK targets in PC3 cells. The expression of p-ERK1/2 and ERK1/2 in the four groups of PC3 cells (BC, NC, or shERβ plasmid [with or without the MEK inhibitor PD98059]) was examined (**Figure 1A** and **1B**). Western blotting data showed that ERK1/2 protein expression was not significantly different among the four groups ($P>0.05$; **Figure 1B**). However, expression of p-ERK1/2 in the shERβ group was significantly higher than that in the other groups ($^*P<0.05$; **Figure 1B**). The expression of p-ERK1/2 in shERβ+ PD98059 group was the lowest compared to that in all other groups ($^\#P<0.05$; **Figure 1B**).

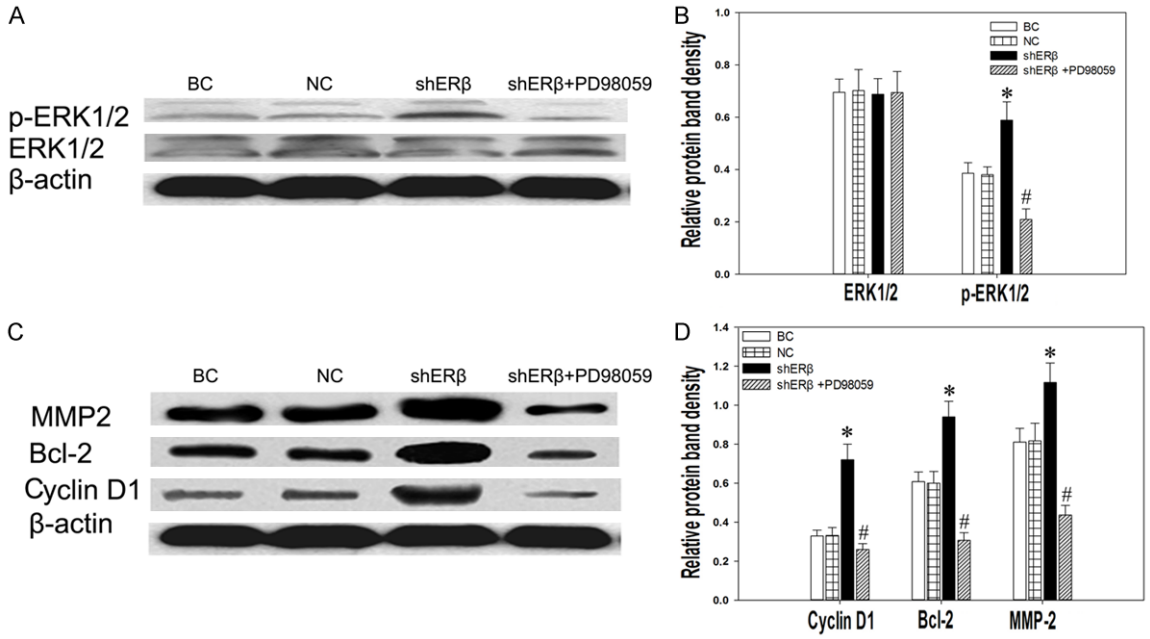


Figure 1. Reduced expression of ERβ activates the p-ERK1/2 signaling pathway. A and B: There was no significant difference of ERK1/2 expression in the four groups. Expression of p-ERK1/2 in the shERβ group is the highest, but in the shERβ+PD98059 group is the lowest. C and D: Expression of cyclin D1, MMP2 and Bcl-2 in the shERβ group is the highest, but in the shERβ+PD98059 group is the lowest. BC, blank control; NC, negative control; shERβ, psilencer-2.1-U6-neo-shERβ group; ERβ+PD98059, psilencer-2.1-U6-neo-shERβ+MEK inhibitor PD98059 group. **P*<0.05, #*P*<0.05.

Western blotting analysis also showed that the expression of cyclin D1, MMP2, and Bcl-2 proteins in the shERβ group was significantly increased, compared to that in other groups (**P*<0.05; **Figure 1C** and **1D**). Cyclin D1, Bcl-2, and MMP2 protein expression in the shERβ+PD98059 group was significantly lower than that in other groups (#*P*<0.05; **Figure 1C** and **1D**). Taken together, these data indicate that silencing of ERβ in prostate cancer cells increases ERK1/2 phosphorylation, without impacting ERK1/2 protein levels, and increases the expression of cyclin D1, MMP-2, and Bcl-2 proteins, in a MEK-dependent manner.

ERβ low-expression increase cell proliferation of PC3 cells

To determine the effect of ERβ silencing on cell proliferation, PC3 cells were transfected with the shERβ expression plasmid, with or without a MEK inhibitor. From flow cytometry analysis, the percentage of cells in the G0/G1 phase in the BC, NC, shERβ, and shERβ+PD98059 groups were 55.38 ± 3.32%, 56.96 ± 3.56%, 35.1 ± 2.47%, and 76.62 ± 3.85%, respectively (**Figure 2A** and **2C**). Decreased expression of ERβ dramatically decreased the proportion of cells in the G0/G1 phase from 55% to 35%. The percentage of cells in the G0/G1 phase in the shERβ group was significantly lower than those in the other three groups (**P*<0.05; **Figure 2C**). The percentage of cells in the G0/G1 phase in the shERβ+PD98059 group was significantly higher than those in the other three groups (#*P*<0.05; **Figure 2C**). These data showed that reducing ERβ expression had a growth-promoting effect.

Decreased ERβ expression decreases cell apoptosis in PC3 cells

Next, we assessed the effect of ERβ silencing on apoptosis. Flow cytometry analysis showed that the percentage of apoptotic cells in the BC, NC, shERβ, and shERβ+PD98059 groups were 60.8 ± 1.9%, 61.5 ± 2.31%, 34.7 ± 2.59%, and 80.3 ± 3.2%, respectively (**Figure 2B** and **2D**). The percentage of apoptotic cells in the shERβ group was significantly lower than those in the other three groups (**P*<0.05; **Figure 2D**). Conversely, the percentage of apoptotic cells in the shERβ+PD98059 group was significantly higher than those in the other three groups

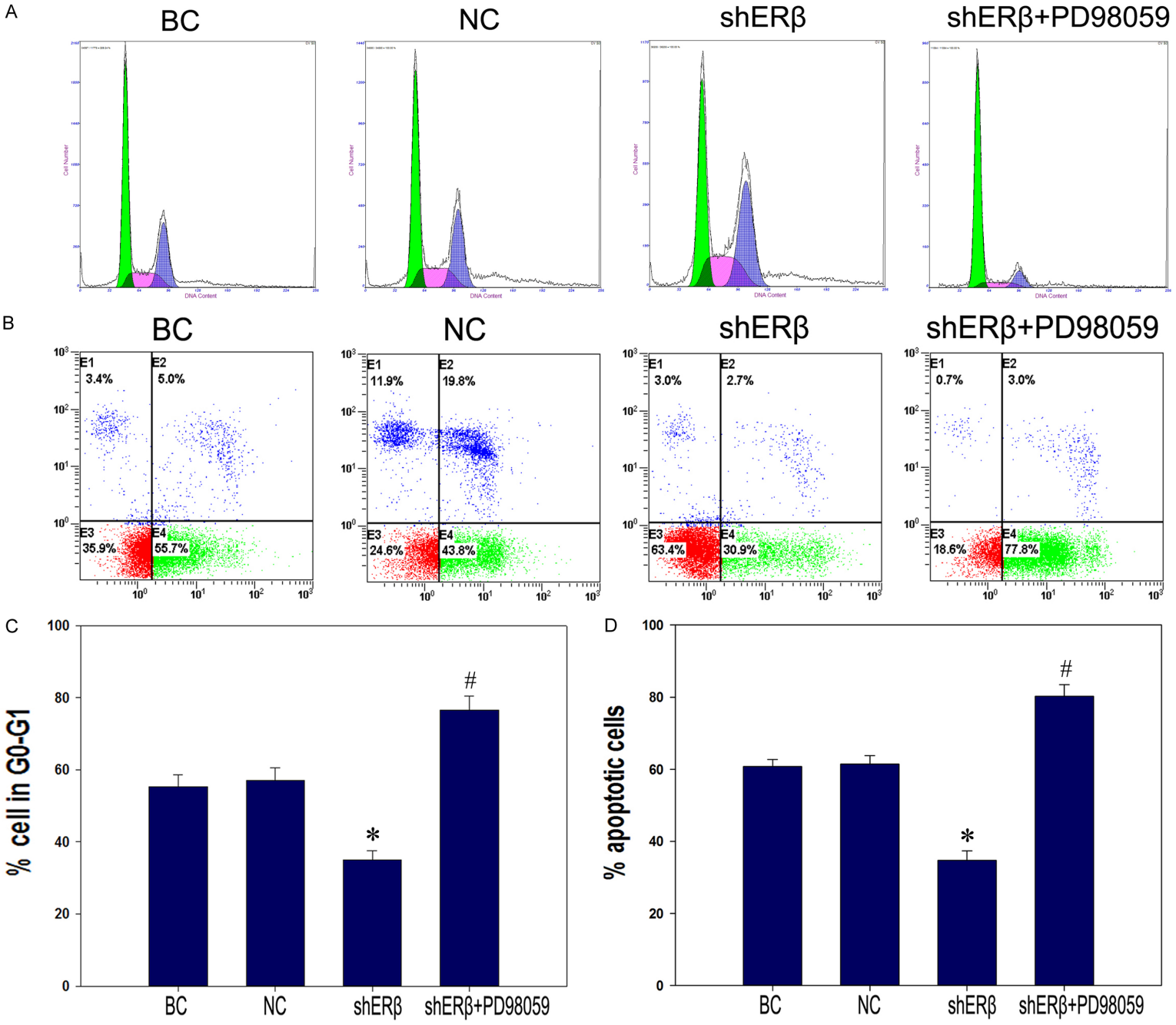

A
BC
NC
shERβ
shERβ+PD98059
B
BC
NC
shERβ
shERβ+PD98059
E1
3.4%
E2
5.0%
E3
35.9%
E4
55.7%
E1
11.9%
E2
19.8%
E3
24.6%
E4
43.8%
E1
3.0%
E2
2.7%
E3
63.4%
E4
30.9%
E1
0.7%
E2
3.0%
E3
18.6%
E4
77.8%
C
% cell in G0-G1
BC
NC
shERβ
shERβ+PD98059
D
% apoptotic cells
BC
NC
shERβ
shERβ+PD98059

Figure 2. ERβ low-expression increase cell proliferation and decreases cell apoptosis of PC3 cells. A and C: Proliferation effect of shERβ on PC3 cells. Histograms show the DNA content of PC3 cells. The percentage of cells in G0/G1 phase of shERβ group is the lowest. B and D: The inducing-apoptosis effect of shERβ on PC3 cells. The histograms show the apoptotic rate of PC3 cells. The percentage of apoptosis cells in the shERβ group is the lowest. BC, blank control; NC, negative control; shERβ, psilencer-2.1-U6-neo-shERβ group; ERβ+PD98059, psilencer-2.1-U6-neo-shERβ+MEK inhibitor PD98059 group. *P<0.05, #P<0.05.

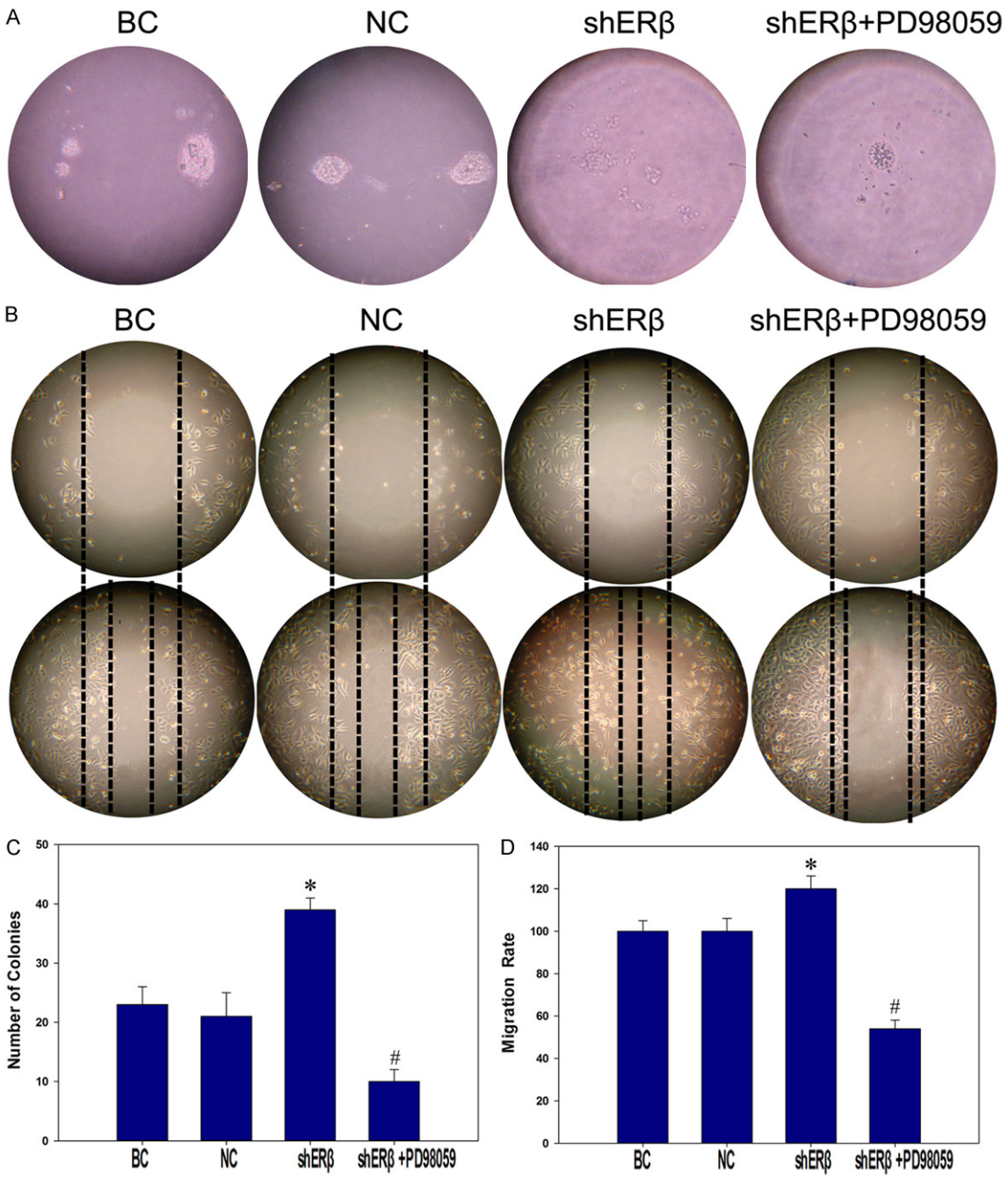


Figure 3. ERβ silencing promotes colony formation and cell migration of PC3 cells. A: Colony-formation assay. B: Cell migration assay. C: shERβ-transfected PC3 cells resulted in a significant increase of the numbers of colonies. D: shERβ-transfected PC3 cells resulted in a significant increase of migration distance. BC, blank control; NC, negative control; shERβ, psilencer-2.1-U6-neo-shERβ group; ERβ+PD98059, psilencer-2.1-U6-neo-shERβ+MEK inhibitor PD98059 group. *P<0.05, #P<0.05.

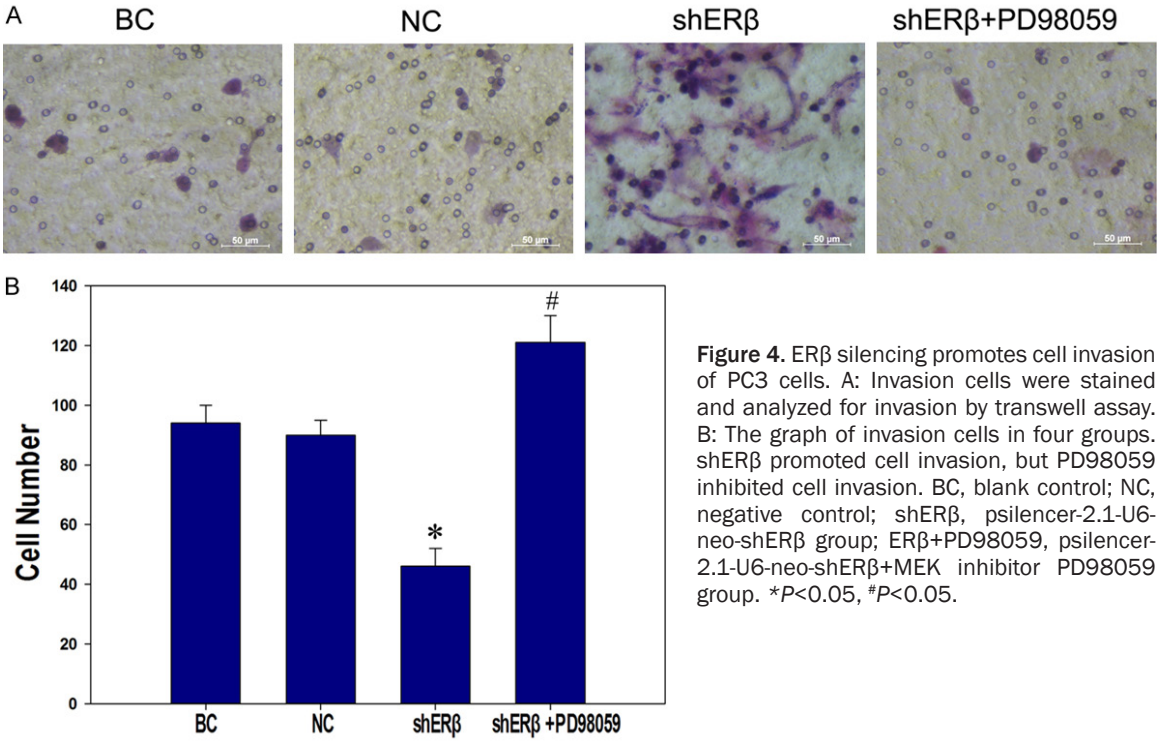


Figure 4. ERβ silencing promotes cell invasion of PC3 cells. A: Invasion cells were stained and analyzed for invasion by transwell assay. B: The graph of invasion cells in four groups. shERβ promoted cell invasion, but PD98059 inhibited cell invasion. BC, blank control; NC, negative control; shERβ, psilencer-2.1-U6-neo-shERβ group; ERβ+PD98059, psilencer-2.1-U6-neo-shERβ+MEK inhibitor PD98059 group. *P<0.05, #P<0.05.

(**Figure 2D**, #P<0.05). These data showed that suppression of ERβ expression via shRNA-mediated methods suppresses apoptosis in prostate cancer cells.

ERβ silencing promotes colony formation in PC3 cells

The ability of cancer cells to form colonies in soft agar is a key indicator of their ability to proliferate. Colony formation assays were carried out to evaluate the ability of the cells to proliferate in the semi-solid agar matrix. The colony counts for the BC, NC, shERβ, and shERβ+PD98059 cells were 23 ± 3, 21 ± 4, 39 ± 2, and 10 ± 2, respectively (**Figure 3A** and **3C**). The shERβ+PD98059 group formed fewer colonies than the cells in the other three groups (*P<0.05; **Figure 3C**), while the shERβ cells formed more colonies than those in the other three groups (#P<0.05; **Figure 3C**). These data suggest that ERβ silencing promotes colony formation in prostate cancer cells.

ERβ silencing promotes migration of PC3 cells

To investigate the effects of ERβ on cell motility, wound-healing assays was performed. The cell migration rate in BC, NC, shERβ, and shERβ+PD98059 cells were 100 ± 5%, 100 ± 6%, 120 ± 6%, and 54 ± 4%, respectively (**Figure 3B** and **3D**). The data show that ERβ silencing increases migration of PC3 cells (*P<0.05; **Figure 3D**); conversely, inhibition of MEK using PD98059 decreases ERβ-mediated augmentation of PC3 cell migration (*P<0.05; **Figure 3D**).

ERβ silencing promotes cell invasion

The regulation of cell invasion is a critical factor that drives cancer progression. To examine the influence of ERβ on the invasiveness of prostate cancer cells, Matrigel transwell invasion assays were carried out. The number of invading cells in the BC, NC, shERβ, and shERβ+PD98059 groups were 94 ± 6, 90 ± 5, 46 ± 6, 121 ± 9, respectively (**Figure 4A** and **4B**). The data showed that ERβ silencing promotes cell invasion after incubation for 24 h (*P<0.05; **Figure 4B**), and that MEK inhibition with PD98059 reversed ERβ silencing-mediated increases in cell invasion (*P<0.05; **Figure 4B**). These data indicate that ERβ silencing promotes cell invasion, and that this effect, akin to the effect of ERβ silencing on cell proliferation and migration, is dependent on MEK activity.

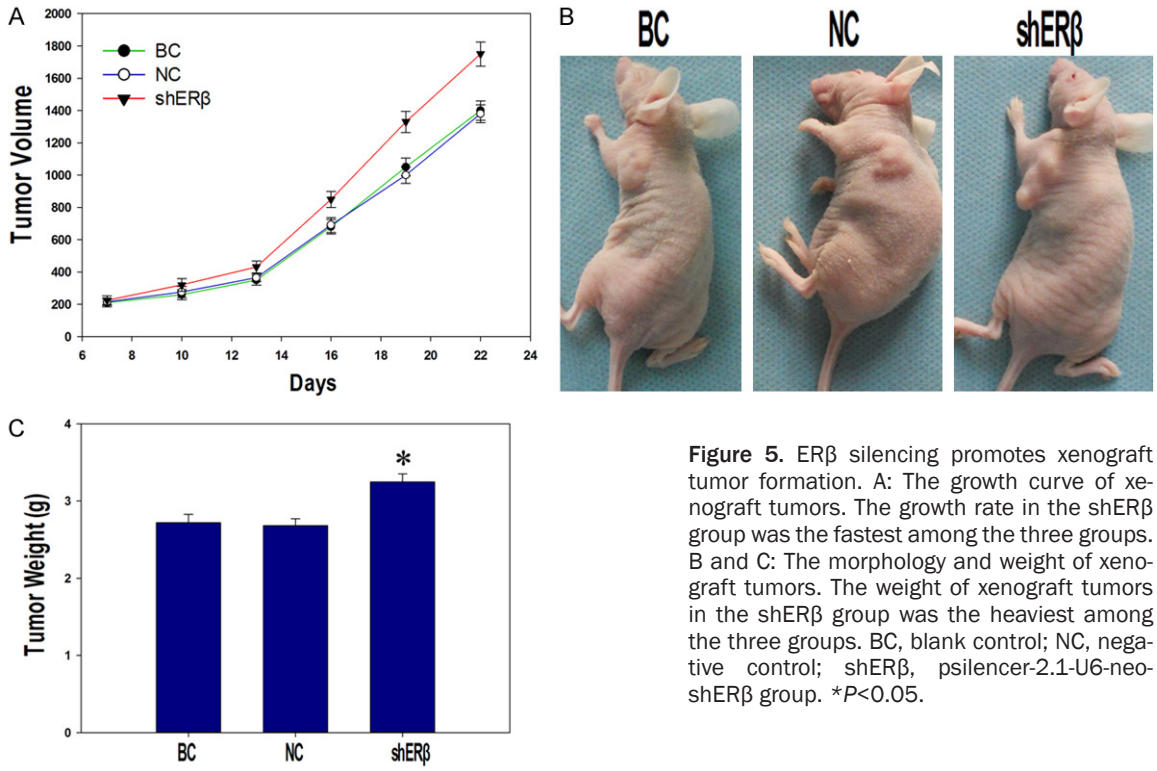


Figure 5. ERβ silencing promotes xenograft tumor formation. A: The growth curve of xenograft tumors. The growth rate in the shERβ group was the fastest among the three groups. B and C: The morphology and weight of xenograft tumors. The weight of xenograft tumors in the shERβ group was the heaviest among the three groups. BC, blank control; NC, negative control; shERβ, psilencer-2.1-U6-neo-shERβ group. *P<0.05.

ERβ silencing promotes tumor formation

To address the effects of ERβ silencing on tumor growth in vivo, we used a tumor xenograft mouse model using PC3 cells stably-transfected with vector alone (BC), scrambled shRNA vector (NC), or ERβ-targeting shRNA (shERβ). The tumor growth rate in mice implanted with shERβ cells was significantly faster than that in the other two groups (**Figure 5A** and **5B**). The weight of the tumors in mice transplanted with the BC, NC, and shERβ cells were 2.72 ± 0.11 g, 2.68 ± 0.09 g, and 3.25 ± 0.10 g, respectively (*P<0.05; **Figure 5C**). Immunohistochemical analysis of tumor tissues showed that the proportion of ERβ-positive cells in tumors from mice transplanted with BC, NC, and shERβ cells were 110 ± 6%, 106 ± 5%, and 35 ± 4%, respectively (**Figure 6A** and **6D**). The expression levels of p-ERK1/2 protein in the BC, NC, and shERβ groups were 16 ± 2%, 17 ± 3%, and 112 ± 5%, respectively (**Figure 6C** and **6F**). There was no significant difference in ERK protein expression between the three groups (*P>0.05; **Figure 6B** and **6E**). However, shRNA-mediated silencing of ERβ promoted phosphorylation of ERK protein in tumor tissues (*P<0.05; **Figure 6F**). Western blotting analysis showed that the expression of VEGF protein in the shERβ group was higher than that in the other two groups (*P<0.05; **Figure 7**). These findings provide evidence for ERβ silencing-mediated promotion of tumor formation by activation of ERK1/2 protein phosphorylation.

Discussion

Prostate cancer is one of the biggest threats to men's health. Many reports have shown that loss of ERβ expression is associated with prostate cancer [23, 24]. Kim IY et al. [25] reported ERβ expression along with loss/reduced expression of ERα in androgen-independent PC3 cells. Pravettoni A et al. [26] reported that different doses of estradiol and selective ER modulators (SERMs) induced a time-dependent inhibition of proliferation in DU145 cells. The preventive and therapeutic effects of SERMs on prostate cancer were also tested in clinical trials. In spite of these trials, successful treatment protocols for advanced prostate cancer using SERMs have not yet been established or reported. Prostate cancer progression is dependent on androgen. Androgen withdrawal therapy is an effective therapy for prostate cancer; by reducing androgen levels, the conver-

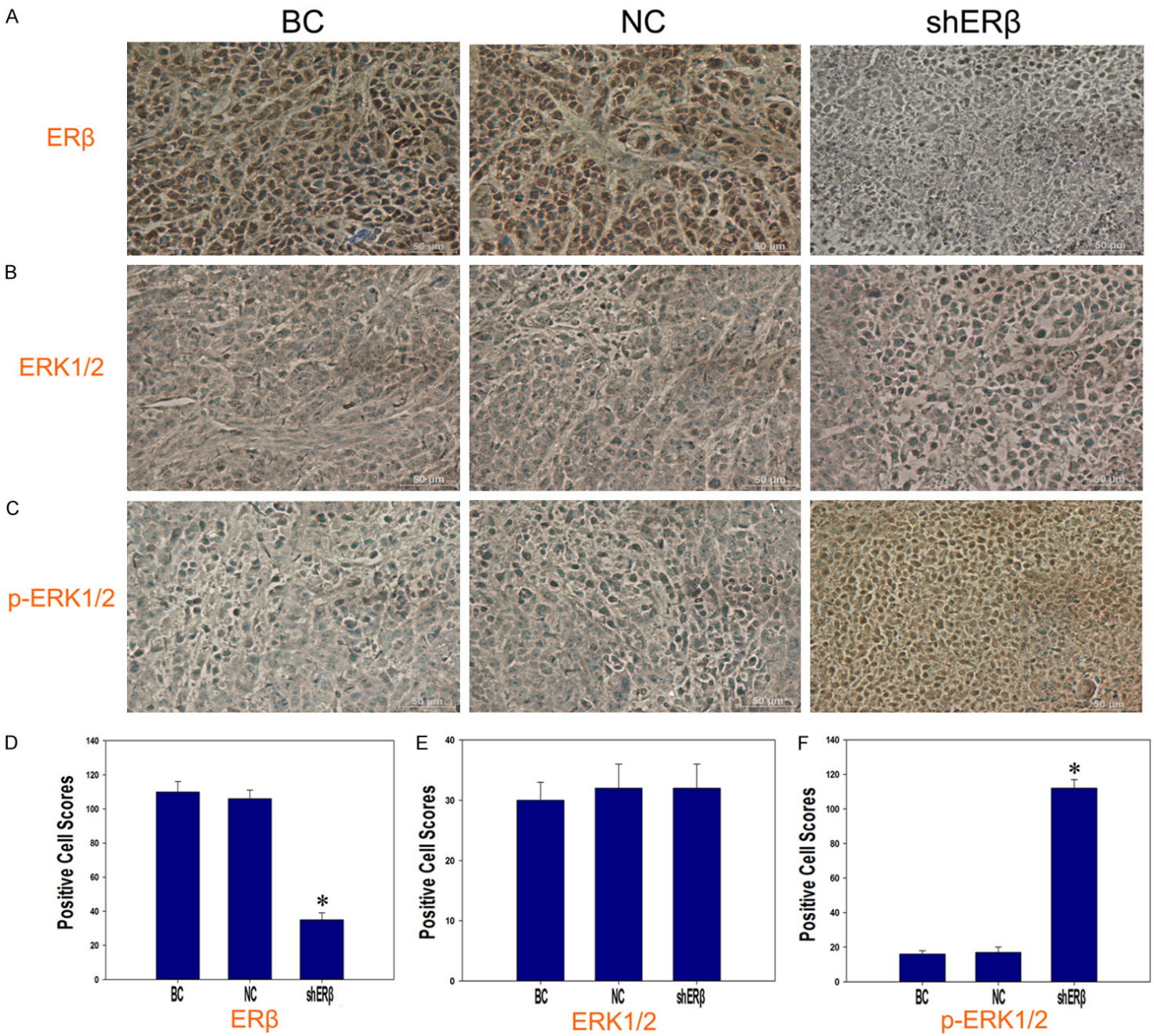
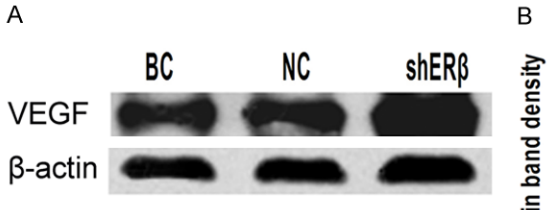

A
BC
NC
shERβ
ERβ
B
ERK1/2
C
p-ERK1/2
50 μm
D
Positive Cell Scores
BC
NC
shERβ
ERβ
E
Positive Cell Scores
BC
NC
shERβ
ERK1/2
F
Positive Cell Scores
BC
NC
shERβ
p-ERK1/2

Figure 6. ERβ silencing promotes tumor formation by activating ERK1/2 expression. A-C: The protein expression of ERβ, ERK1/2, and p-ERK1/2 was detected in xenograft tumor tissue of three groups by immunohistochemistry (×400). D: ERβ positive expression in the shERβ group is the lowest. E: There was no significant difference of ERK1/2 positive expression. F: p-ERK1/2 positive expression in the shERβ group is the highest. BC, blank control; NC, negative control; shERβ, psilencer-2.1-U6-neo-shERβ group. *P<0.05.



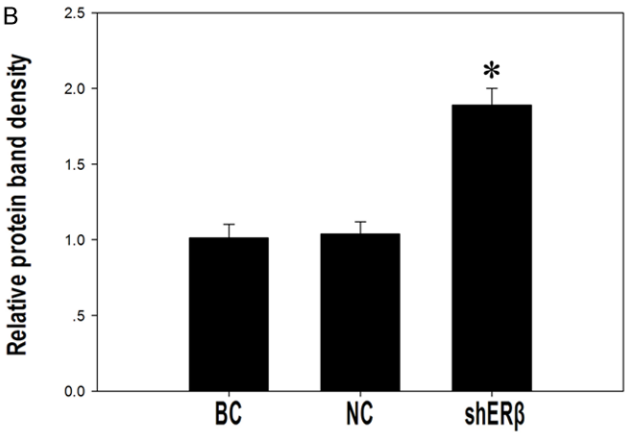


Figure 7. ERβ silencing enhances VEGF protein expression in xenograft tumor tissue. A: Western blot analysis of VEGF protein expression in xenograft tumor tissue. B: VEGF protein expression in the shERβ group was the highest in the three groups. BC, blank control; NC, negative control; shERβ, psilencer-2.1-U6-neo-shERβ group. *P<0.05.

sion of testosterone to dihydrotestosterone is inhibited, thereby inhibiting the growth of prostate cancer cells. However, it has been difficult to achieve satisfactory therapeutic effects in patients with androgen-independent prostate cancer, so more effective treatment methods are needed, especially in patients with this subtype of prostate cancer.

The ERK1/2 signaling pathway is one of the classical Ras-Raf-MEK-ERK signal transduction pathways. It plays important roles in cell division, cell migration, and tumor invasion, and participates in the signal transduction of various cytokines, as well as promoting mitosis and hormone receptor signal transduction in the cell [9-11]. Continuous activation of ERK1/2 exists commonly in lung, breast, and ovarian cancers, and in other malignant tumor cells [12-14]. One report showed that the occurrence of prostate cancer is closely related to the activation of the ERK1/2 pathway, and that the activation of the ERK1/2 pathway promotes acquisition of androgen-independence characteristics in prostate cancer cells [15]. To elucidate the molecular mechanism underlying ERβ and the ERK1/2 pathway, we monitored the total and activated (phosphorylated) forms of ERK1/2 proteins, in the presence or absence of the MEK inhibitor PD98059. Our data showed that, while there was no significant difference in total ERK1/2 expression among the treatment groups, p-ERK1/2 expression was highest in cells in which ERβ expression was silenced and lowest in ERβ-silenced cells treated with PD98059. These data suggest that decreased expression of ERβ results in increased phosphorylation of ERK1/2 protein. In the in vivo experiments carried out in the mouse tumor xenograft model, the no significant differences in ERK1/2 expression were noted between the three groups. Consistent with the data in cultured cells, p-ERK1/2 expression was highest in tumors from mice transplanted with shERβ cells. These results further confirmed that the positive regulatory effect of shERβ on ERK1/2 signaling pathway.

Flow cytometry was carried out to evaluate the effect of ERβ gene silencing on proliferation and apoptosis of androgen-independent PC3 prostate cancer cells. The results of the cell cycle analysis confirmed that ERβ gene silencing promotes cell mitosis and leads to cell proliferation, and that this effect can be inhibited by PD98059, an ERK1/2 signaling pathway inhibitor. Moreover, the highest proportion of apoptotic cells was detected in the shERβ+ PD98059 group, while the lowest proportion was detected in the shERβ group. These data suggest that ERβ expression helps regulate cell proliferation in PC3 cells.

Many proteins are involved with the progression of cell proliferation and apoptosis. Cyclin D1, a key cell cycle regulator, alters the pro-

gression of the cell cycle. Cyclin D1 overexpression has been found in many tumors and might contribute to tumorigenesis [27]. The Bcl-2 gene is a proto-oncogene the product of which can inhibit apoptosis [28]. Four main mechanisms of Bcl-2-mediated anti-apoptosis have been described: inhibiting cytochrome c (a pro-apoptotic factor) release from mitochondria to cytoplasm; antagonizing pro-apoptotic Bax gene expression; suppressing oxidation and maintaining intracellular Ca^{2+} homeostasis; and blocking cytochrome c in cytoplasm to activate caspase [29-31]. In this study, the expression of Bcl-2 and cyclin D1 proteins was highest in the shERβ group and lowest in the shERβ+PD98059 group. These results demonstrate that silencing of ERβ expression can promote cell proliferation and inhibit apoptosis, partly by increasing cyclin D1 and Bcl-2 protein expression.

Tumor progression requires enhanced invasion and metastasis. In this study, we utilized wound-healing, soft agar colony formation, and transwell invasion assays to assess the impact of ERβ on invasion and migration of PC3 cells. The data showed that colony formation, cell invasion, and cell migration were suppressed in cells with shRNA-mediated stable silencing of ERβ, and that all these effects were reversible upon inhibition of the ERK1/2 pathway using the MEK inhibitor PD98059. These data provide string evidence for the role of ERβ in maintaining/modulating cell differentiation and suppressing migration and invasion of prostate cancer cells.

Previous research has shown that cell adhesion factors, angiogenesis factors, and extracellular matrix proteins are correlated with tumor formation and invasion [32]. Expression of MMPs is increased in tumors and these enzymes are key players in facilitating turnover of the extracellular matrix, thereby promoting tumor invasion and metastasis [33]. MMP-2, a member of the MMP family, is thought to acts as a drill, which can facilitate degradation of the collagen matrix and basement membrane, thereby aiding cancer cell escape, tumor migration, and invasion [34, 35]. VEGF is another key factor that drives tumor progression, by participating in angiogenesis and activating endothelial cell proliferation [36]. In this study, we investigated the expression of MMP-2 and VEGF proteins in the background of shRNA-mediated silencing of ERβ in PC3 cells. Our data showed that ERβ silencing results in increased MMP-2 and VEGF protein expression. Taken together, our data suggest that ERβ gene silencing promotes many malignant biological behaviors of PC3 cell, by activating the ERK1/2 signaling pathway and increasing the protein expression of MMP-2, VEGF, cyclin D1, and Bcl-2. In conclusion, ERβ may play a protective role against malignant progression of androgen-independent prostate cancers. Further studies are warranted to address the utility of ERβ-targeted therapies in management of androgen-independent prostate cancer.

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Disclosure of conflict of interest

None.

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