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Original Article

Effect of ERβ-regulated p38 signaling pathway on biological behaviors of prostate cancer cells

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Abstract: The study is aim to examine the effect of ERβ on the prostate cancer. We investigated several proliferation and apoptosis related genes in protein expression levels in vivo and in vitro. The anti-tumorigenic ability of ERβ was investigated to assess the effect of ERβ on the biological behaviors of LNCaP cells. Real-time RT-PCR and western blot showed that there was highest expression of p-p38 protein in pCDNA-3.1-ERβ group and lowest expression in pCDNA-3.1-ERβ+SB203580 group. There is no significant difference of p38 expression among the four groups. The expression of cyclin D1, Bcl-2 and MMP2 were highest in pCDNA-3.1-ERβ+SB203580 and lowest in pCDNA-3.1-ERβ group. Flow cytometry showed that ERβ could repress cell proliferation by increasing the percentage of cells in G0/G1 phases. Soft colony forming experiments and transplantation tumor mode showed that ERβ could inhibit the tumor formation. Scratch experiments and transwell invasion experiment suggested that the number of migration cells was highest in pCDNA-3.1-ERβ+SB203580 group and lowest in pCDNA-3.1-ERβ group. In addition, immunohistochemical results showed that ERβ protein were positive expression in pCDNA-3.1-ERβ xenograft tumor and vascular endothelial growth factor (VEGF) was low expression compared to other groups. These results suggested that ERβ can inhibit malignant biological behavior of prostate cancer LNCaP cell by activating p38 signaling pathways and promote cell differentiation by inhibiting the expression of VEGF protein.

Keywords: Estrogen receptor beta, p38, proliferation, apoptosis, prostate cancer

Introduction

Prostate cancer (PCa) is one of the most common malignant diseases in aging men [1]. The prostate malignancy can be affected by sex hormone, including estrogen, androgen and progesterone. Estrogens may have opposite effects on cell proliferation, as demonstrated in many experimental models [2, 3]. A growing body of evidence has proved that the protective role played by ERβ against uncontrolled cell proliferation in human breast cancer [4, 5]. In particular, the activation of this receptor subtype is linked to increased differentiation and inhibition of cell proliferation [6]. However, the mechanism of the ERβ overexpression in prostate cancer cells is not clear.

MAPKs as a group of serine/threonine protein kinases are the smallest molecules in the inhibitor of apoptosis proteins (IAP) family, and promote cell survival via activation of diverse signal-transduction pathways. p38 signal pathway as one of the MAPKs signaling pathways is one of main regulating apoptosis pathway [7]. In humans, ERβ expression gradually disappears in the progress of prostate cancer. ERβ expression was strong in human normal prostate epithelial, but at low level in intraepithelial neoplasia [8, 9]. ERβ may have a positive role in the inhibition of cell proliferation and the low expression of ERβ in prostate cancer may be in the process of prostate cancer cells escape. However, the molecular mechanism of ERβ regulating p38 signal pathway is not clear.

Therefore, the main purpose of the study was to assess some of the possible molecular mechanisms of ERβ in prostate tumorigenesis. We have evaluated the effect of ERβ on cell prolif-

eration and tumor formation ability. In addition, the effect of ERβ on p38 signal pathway was discussed. Our studies also demonstrate the mechanism that ERβ induce prostate tumorigenesis by activating p38.

Materials and methods

Cell culture and transfection

Human prostate cancer LNCaP cell line was obtained from American Type Culture Collection and cultured according to the manufacturer's instructions. Cells were transfected (at 80% confluence) using Lipofectamin 2000 (Invitrogen, CA) according to the manufacturer's instructions. This study included four groups: blank-control group (BC group, transfection with mock), negative-control group (NC group, transfection with pCDNA3.1(-)), pCDNA-3.1-ERβ group (ERβ group, transfection with pCDNA-3.1-ERβ), and pCDNA-3.1-ERβ+SB203580 group (ERβ+SB203580 group, incubated with p38 MAPK inhibitor SB203580 after transfection of pCDNA3.1(-)-ERβ plasmid). Cells were harvested at 48 hours after transfection for further analysis.

Plasmids construction

Full length ERβ gene was amplified from human ovarian tissue using the primers 5'-GGTCTAGAGCTGTTATCTCAAGACATGGATATAA-3' and 5'-TAGGATCCGTCACTGAGACTGTGGGTTCTG-3', and subsequently cloned into the *Xba*I and *Bam*HI sites of the pcDNA3.1(-) vector. Recombination plasmid was confirmed by sequencing.

Real-time quantitative RT-PCR

Total RNA was extracted from cells using Trizol Reagent (Invitrogen, USA) and incubated with DNase I to remove genomic DNA. Then, cDNA synthesis was performed using MMLV reverse transcriptase (Promega, USA) according to the manufacturer's protocol.

SYBR green-based real-time quantitative RT-PCR was performed on the iCycler System (Bio-Rad, USA). The β-actin gene was used as a normalization control. The sequences of β-actin gene amplification were 5'-CTGCGTCTGGACCTGGATGG-3' for forward primer and 5'-CGATGGTGATGACCTGGCTGT-3' for reverse primer. The sequences of ERβ gene amplification were: 5'-AGAGTCCCTGGTGTGAAGCAA-3' for forward primer and 5'-GACAGCGCAGAA GTGAGCATC-3' for reverse primer.

Western blot

Western blot was carried out according to one described earlier report [10]. Samples were immunoblotted with rabbit polyclonal antibody anti-p38 (Ab31242, Abcam, USA), anti-p-p38 (ab4822, Abcam, USA), anti-VEGF (ab46154, Abcam, USA) anti-ERβ (ab3576, Abcam, USA), anti-caspase-3 (ab90437, Abcam, USA) and anti-Bcl-2 (ab18210, Abcam, USA). Rabbit monoclonal antibodies anti-cyclin D1 (ab16663, Abcam, USA) and anti-MMP2 (ab51125, Abcam, USA) followed by a further incubation with secondary antibody (#7074, Cell Signaling, USA). Monoclonal antibody anti-β-actin-peroxidase (A3854, Sigma-Aldrich, USA) was used as a normalization control.

Flow cytometry (FCM) analysis

For apoptosis measurement, the number of apoptotic cells was measured by flow cytometry using an Annexin-V FITC/propidium iodide (PI) apoptosis detection kit (Promega, USA) according to the manufacturer's protocol. Cell cycle was evaluated by using the PI staining method.

Colony formation assay

Soft agar dishes were prepared as described previously [11]. LNCaP cells were transfected with either pcDNA3.1(-) or pcDNA3.1(-)-ERβ for 48 hours and allowed to grow until visible colonies formed (14 days). Colonies are counted with a microscope (Olympus CH-40; Japan). Colonies that were greater than 2 mm in diameter were counted. Untransfected LNCaP cells were served as blank control.

Tumor xenograft assay

Tumor xenograft assay was performed as our previous study [12]. LNCaP cells were transfected with either pcDNA3.1(-) or pcDNA3.1(-)-ERβ plasmids, and maintained in the presence of 400 mg/ml of G418 after transfection for 48h. After 3-4 weeks of selection, G418-resistant colonies were isolated. For in vivo experiment, 6×10^7 cells of the resistant cells were injected into the flank of three 4-5-week-

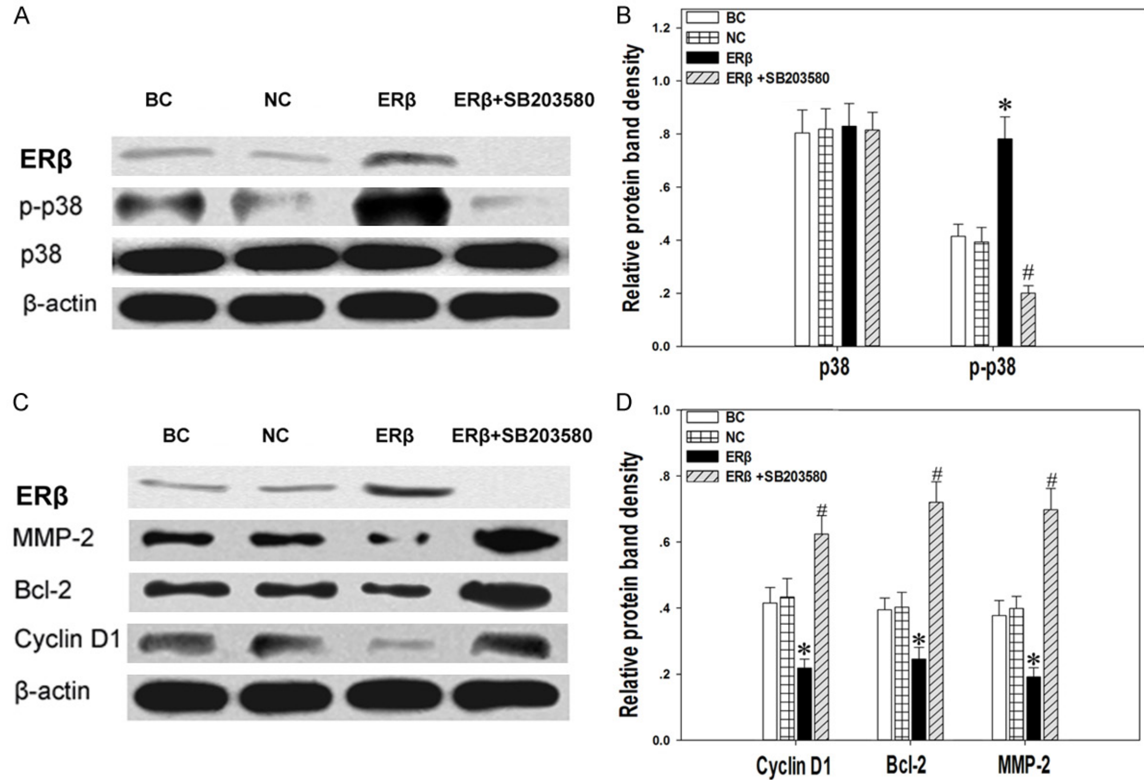

Figure 1. ERβ could activate the expression of protein in p38 signal pathway. A and B. Western blot analysis of ERβ, p38 and p-p38 protein expression. Bars represent the intensity of the bands quantitated by densitometry. C and D. Western blot analysis of the expression of ERβ, Bcl-2, Cyclin D1 and MMP-2 protein. Bars represent the intensity of the bands quantitated by densitometry. BC, blank control; NC, negative control; ERβ, pCDNA-3.1-ERβ group; ERβ+SB203580, pCDNA-3.1-ERβ plus p38 inhibitor SB203580 group. $^{*}P<0.05$, $^{\#}P<0.05$.

old female Balb/c nude mice (Experimental Animal Center of the Hunan province, China). Mice were monitored at periodic intervals (three days). After 22 days, mice were sacrificed and the tumors were analyzed.

In vitro migration/wound healing assay

After 24 h of transient transfection, cells were grown to confluence in a 24-well plate. The confluent monolayer cells was scraped using a sterile pipette tip between two parallel lines and then washed three times with PBS to remove cell debris. Wounds were photographed at 0 h and 24 h after wounding. The migration ability of the cells was evaluated by measuring the width of the wounds at 0h and 24 h for LNCaP cells. The migration rate is described as a percentage compared to the control group.

Matrigel invasion assay

Invasion ability of ERβ gene was determined using 8-μm Matrigel invasion membrane (Corning, USA). The transwell membrane was freshly coated with Matrigel. At 24 h after transient transfection, cells were seeded into upper chamber in serum-free medium. Lower chamber were filled with medium containing 20% FBS. Cells were incubated at 37°C with 5% CO_2 for 24 h, and then non-invading cells were removed by swabbing the top layer of Matrigel. The membranes containing invading cells were fixed with methanol followed by staining with May-Grunwald and Gimesa. The invasion cells were counted and photographed under a microscope (Olympus CH-40; Olympus, Japan) at ×200 magnification. Five fields were counted per filter in each group, and the experiment was conducted in triplicate.

Statistical analysis

Data are given as mean ± standard deviation. Data was statistically analyzed using SPSS version 17.0 (Chicago, IL, USA). Analysis of variance (ANOVA) and t-test were widely used to compare group means. A *P*-value of less than 0.05 was considered to be statistically significant.

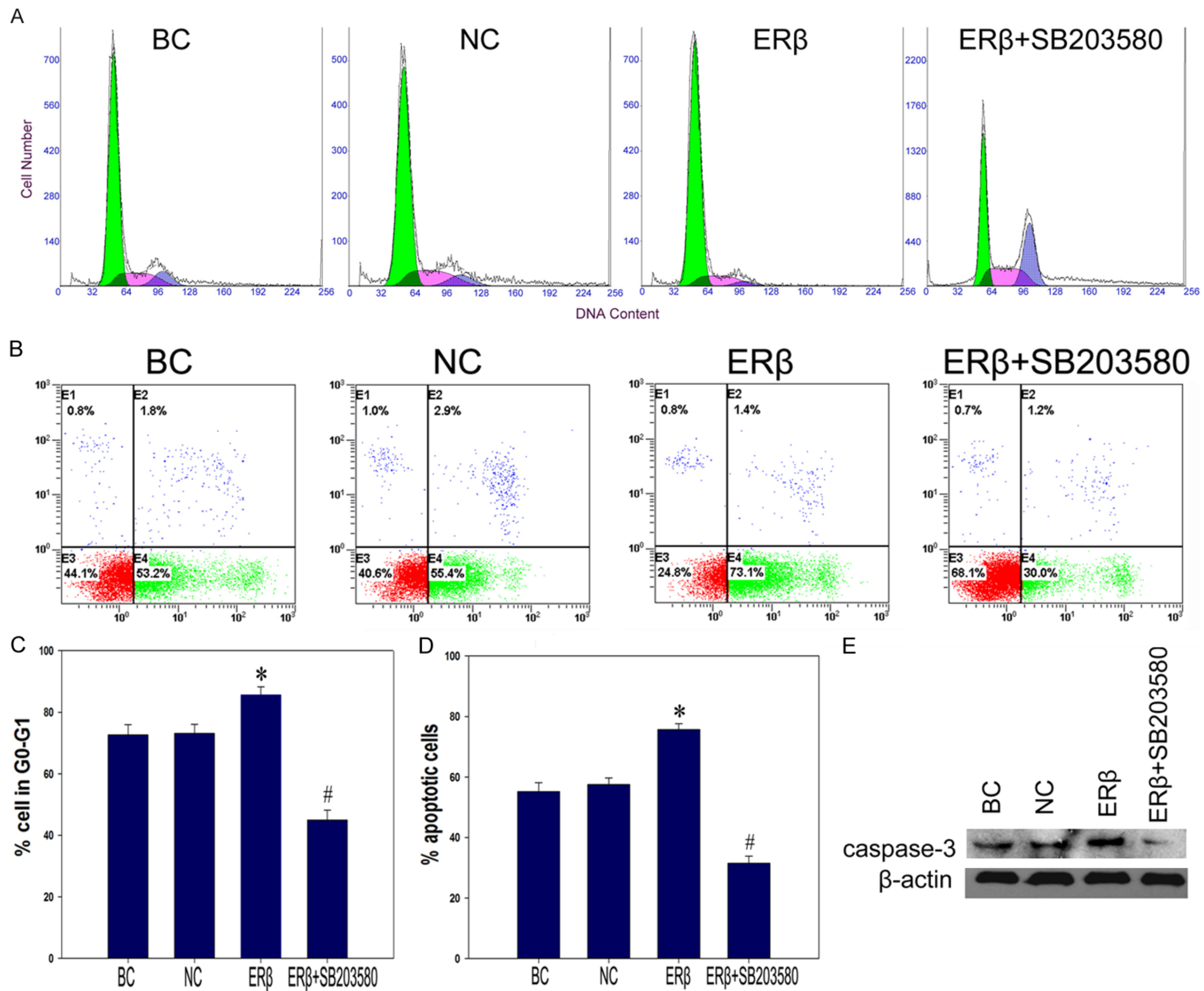
A
BC
NC
ERβ
ERβ+SB203580
Cell Number
DNA Content
B
BC
NC
ERβ
ERβ+SB203580
E1 0.8%
E2 1.8%
E3 44.1%
E4 53.2%
E1 1.0%
E2 2.9%
E3 40.6%
E4 55.4%
E1 0.8%
E2 1.4%
E3 24.8%
E4 73.1%
E1 0.7%
E2 1.2%
E3 68.1%
E4 30.0%
C
% cell in G0-G1
BC
NC
ERβ
ERβ+SB203580
D
% apoptotic cells
BC
NC
ERβ
ERβ+SB203580
E
BC
NC
ERβ
ERβ+SB203580
caspase-3
β-actin

Figure 2. Anti-proliferative effect of ERβ on LNCaP cell growth. A and C. Anti-proliferation effect of ERβ on cell cycle of LNCaP cells. Histograms showing cell the DNA content (PI fluorescence) of LNCaP cells. B and D. The inducing-apoptosis effect of ERβ. Representative FCM images show FITC Annexin V and PI double staining of LNCaP cells. The histograms showing the apoptotic rate of LNCaP cells. E. Western blot analysis of the expression of caspase-3 protein. Three independent experiments were carried out. $^{*}P<0.05$, $^{\#}P<0.05$.

Results

ERβ overexpression activate the p38 MAPK pathway by inhibiting the protein expression of cyclinD1, Bcl-2 and MMP2

LNCaP cells were transfected with mock, pCDNA3.1(-), pCDNA3.1(-)-ERβ plasmid or incubated with p38 MAPK inhibitor SB203580 after pCDNA3.1(-)-ERβ transfection. Different expression level of p38 and p-p38 protein in four experiment groups was observed in LNCaP cells. Western blot showed that there were no significant differences in the level of p38 protein expression among the four groups. However, the expression of p-p38 protein in pCDNA3.1(-)-ERβ group was higher than three other groups significantly (**Figure 1A** and **1B**, $^{*}P<0.05$). The expression of p-p38 in SB203580 group was lower than three other groups significantly (**Figure 1A** and **1B**, $^{\#}P<0.05$).

Western blot showed that the expression of cyclinD1, Bcl-2 and MMP2 in pCDNA3.1(-)-ERβ group was significantly lower than three other groups respectively, as shown in **Figure 1C** and **1D** ($^{*}P<0.05$). The protein expression of cyclinD1, Bcl-2 and MMP2 in SB203580 group was higher than three other groups significantly (**Figure 1C** and **1D**, $^{\#}P<0.05$).

ERβ overexpression decrease cell proliferation of LNCaP cells

To determine the anti-proliferative effect of ERβ, LNCaP cells were transfected with ERβ expression plasmid or incubated with ERβ inhibitor. From FCM analysis, the percentage of cells in the G0/G1 phase in blank control group, negative control group, pCDNA-3.1-ERβ and pCDNA-3.1-ERβ+SB203580 group were 72.67±3.36(%), 73.13±2.98(%), 85.6±2.65(%) and 45.0±3.2(%) respectively. ERβ gene dramatically increased the cells percentage in G0/G1 phase from 73% to 85% in **Figure 2A**. The fractions of LNCaP cells in the G0/G1 phase of the cell cycle in pCDNA-3.1-ERβ group increased compared to other three groups significantly (**Figure 2C**, $^{*}P<0.05$). The fractions of LNCAP cells in the G0/G1 phase of the cell cycle in pCDNA-3.1-ERβ+SB203580 group decreased significantly compared to other three groups ($^{\#}P<0.05$). These data showed that ERβ overexpression had a growth-inhibited effect, but not in control.

ERβ overexpression increase cell apoptosis of LNCaP cells

FCM analysis showed that the percentage of apoptosis cells in blank control group, negative control group, pCDNA-3.1-ERβ and pCDNA-3.1-ERβ+SB203580 group were 55.2±2.98(%), 57.6±2.11(%), 75.7±1.96(%) and 31.5±2.3(%) (**Figure 2B**). Statistically significant ($^{*}P<0.05$) increases of apoptosis cells in pCDNA-3.1-ERβ were observed compared with other three groups (**Figure 2D**). Conversely, statistically significant ($^{\#}P<0.05$) decreases of apoptosis cells in pCDNA-3.1-ERβ+SB203580 group were observed in **Figure 2D**. In addition, the protein expression of caspase-3 in ERβ-transfected cells is higher than other three groups (**Figure 2E**). These data showed that ERβ overexpression had an apoptosis-activated effect compared to control.

ERβ overexpression inhibit colony formation of LNCaP cells

To investigate the role of ERβ overexpression in LNCaP cell growth or survival, colony formation assay were carried out. As shown in **Figure 3A** and **3C**, the colonies in blank control group, negative control group, pCDNA-3.1-ERβ and pCDNA-3.1-ERβ+SB203580 group were 20.0±2.0, 19.0±3.0, 8.0±2.0 and 49.0±4.0 respectively (P<0.05). The colonies in pCDNA-3.1-ERβ+SB203580 group were increased compared to control cells (P<0.05). However, the colonies in pCDNA-3.1-ERβ group was the lowest compared to other three groups (P<0.05). These data demonstrated that ERβ overexpression inhibited colony formation.

ERβ overexpression inhibited migration of LNCaP cells

Wound healing assay was carried out to study the effects of ERβ expression on cell motility.

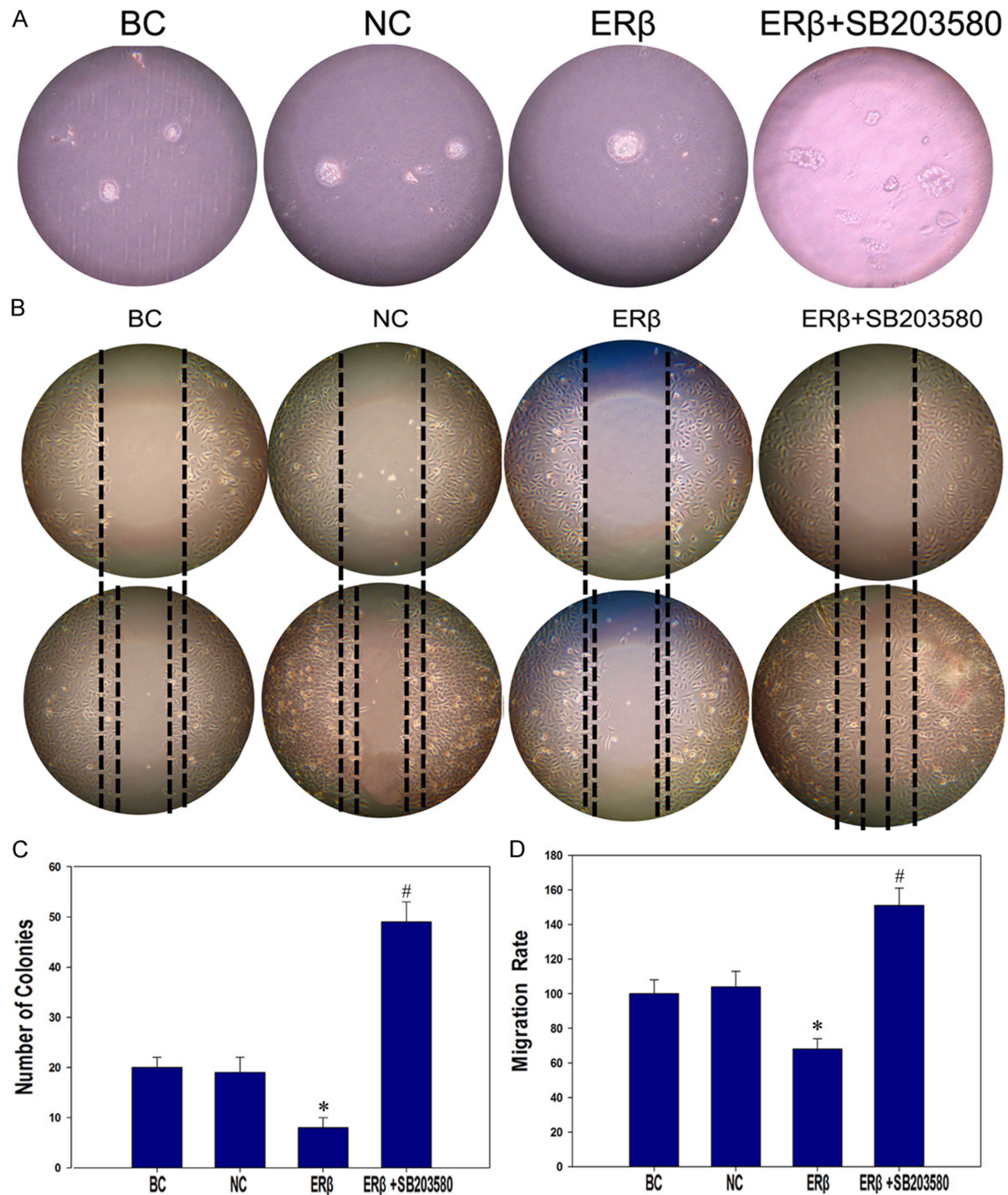

Figure 3. Tumorigenic effect of ERβ on LNCaP cells. A. Colony formation assay. ERβ-transfected LNCaP cells led to a significant decrease of colony numbers compared with the control-transfected cells. The graph shows the mean ± s.d. B. Cell migration assay. ERβ-transfected LNCaP cells decreased the migration of LNCaP cells. C. Colony number based on triplicate independent experiments. D. Migration distance based on triplicate independent experiments. BC, blank control; NC, negative control; ERβ, pCDNA-3.1-ERβ group; ERβ+SB203580. $^*P<0.05$, $^\#P<0.05$.

The migration rate in BC group, NC group, ERβ and ERβ+SB203580 group were 100%±8%, 104%±9%, 68%±6%, and 151%±10% respectively. Wound healing assay suggested that ERβ decreased migration of LNCaP cells, conversely, SB203580 increased migration of LNCaP cells (**Figure 3B** and **3D**, P<0.05).

ERβ overexpression inhibited cell invasion

Transwell invasion assay were performed to examine the invasion ability of ERβ in prostate cancer cell. The invaded cell numbers in blank control group, negative control group and pCDNA-3.1-ERβ and pCDNA-3.1-ERβ+SB203580

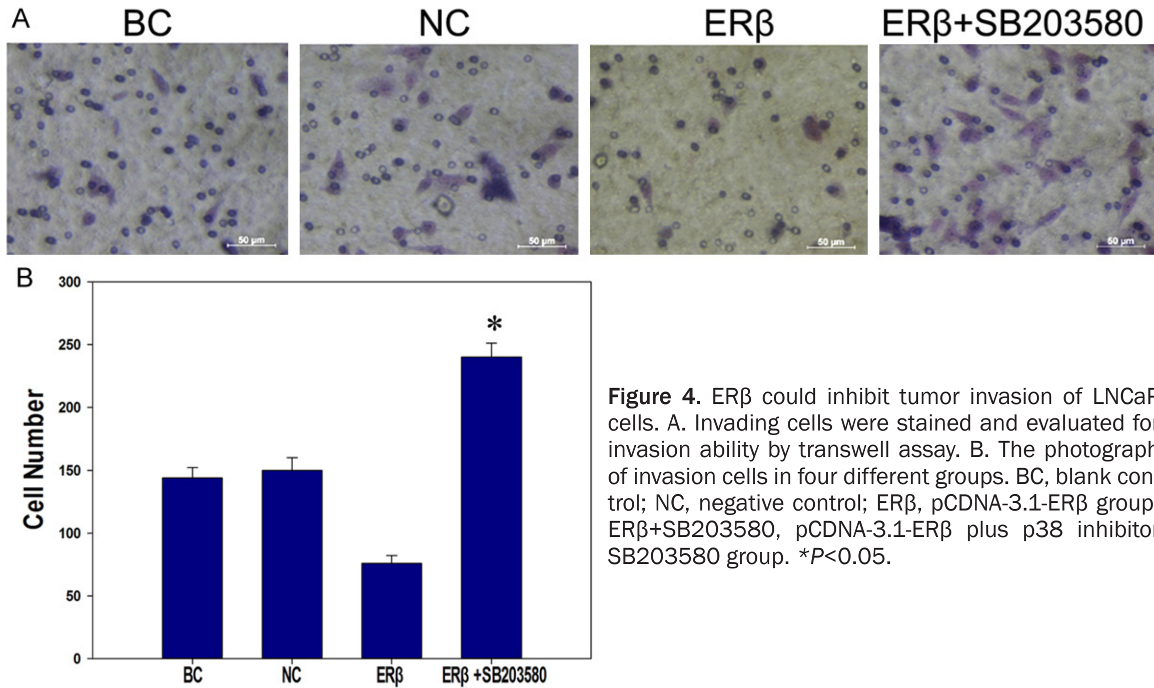

Figure 4. ERβ could inhibit tumor invasion of LNCaP cells. A. Invading cells were stained and evaluated for invasion ability by transwell assay. B. The photograph of invasion cells in four different groups. BC, blank control; NC, negative control; ERβ, pCDNA-3.1-ERβ group; ERβ+SB203580, pCDNA-3.1-ERβ plus p38 inhibitor SB203580 group. *$P<0.05$.

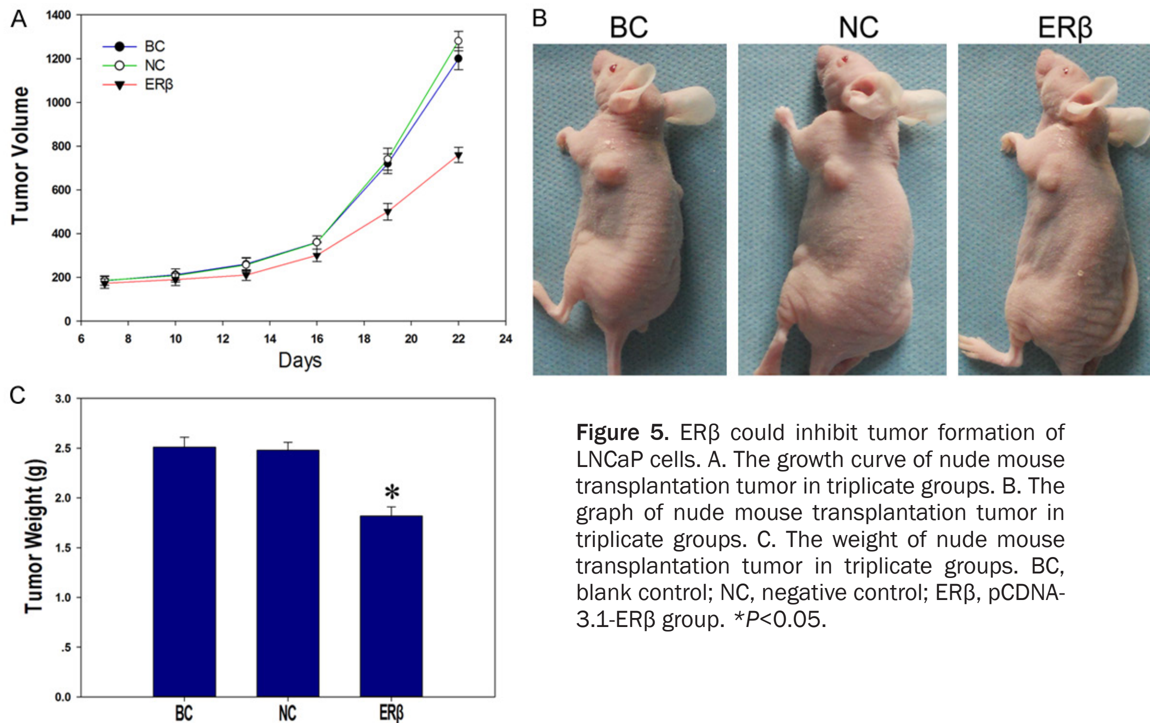

Figure 5. ERβ could inhibit tumor formation of LNCaP cells. A. The growth curve of nude mouse transplantation tumor in triplicate groups. B. The graph of nude mouse transplantation tumor in triplicate groups. C. The weight of nude mouse transplantation tumor in triplicate groups. BC, blank control; NC, negative control; ERβ, pCDNA-3.1-ERβ group. *$P<0.05$.

group were 144±8, 150±10, 76±6 and 240±11 respectively. Therefore SB203580 markedly promoted cell invasion after 24 h incubation (**Figure 4**, P<0.05), however, ERβ inhibited cell invasion.

ERβ overexpression inhibited tumor formation

The volume of tumor in blank control group, negative control group and pCDNA-3.1-ERβ group were 1300 mm^3, 1200 mm^3 and 780

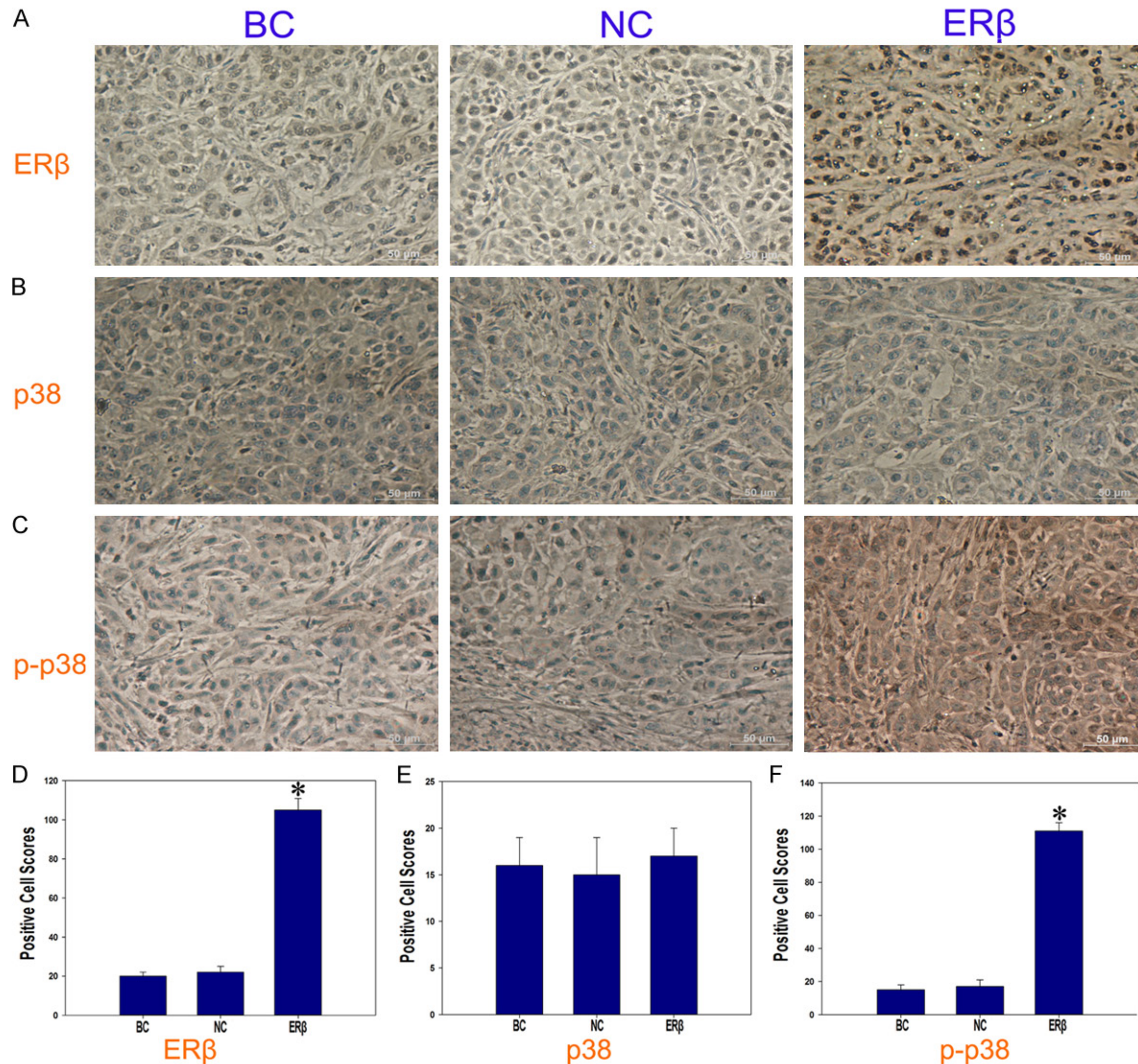

Figure 6. ERβ could inhibit tumor formation by activating p38 expression. A. The ERβ protein expression was examined in three xenograft tumor tissues (×400). B. The protein expression of p38 was examined in three xenograft tumor tissues (×400). C. The protein expression of p-p38 was examined in three xenograft tumor tissue (×400). D. The evaluation and analysis of ERβ positive expression. E. The evaluation and analysis of p38 positive expression. F. The evaluationand analysis of p-p38 positive expression. BC, blank control; NC, negative control; ERβ, pCDNA-3.1-ERβ group. **P*<0.05.

mm^3 respectively. The growth rate in pCDNA-3.1-ERβ group was significantly slower than the other two groups (**Figure 5A** and **5B**, P<0.05). The weight of tumor in BC group, NC group and pCDNA-3.1-ERβ group were 2.51±0.10 g, 2.48±0.08 g and 1.82±0.09 g respectively (**Figure 5C**, P<0.05). IHC result described that the positive expression rate of ERβ protein in BC group, NC group and pCDNA-3.1-ERβ group were 20±2(%), 22±3(%) and 105±6(%) (**Figure 6A** and **6D**, P<0.05). The p38 protein expression level in blank control group, negative control group and pCDNA-3.1-ERβ group were 16±3(%), 15±4(%) and 17±3(%) respectively (**Figure 6B** and **6E**, P<0.05). The result showed that there was no significant expression change of p38 protein. However, the expression level of p-p38 protein in blank control group, negative group and pCDNA-3.1-ERβ group were 15±3(%), 17±4(%) and 111±5(%) (**Figure 6C** and **6F**, P<0.05). ERβ overexpression caused the increased phophorylation of p38 protein. Western blot suggested that the VEGF protein expression in pCDNA-3.1-ERβ group was lower than control groups (**Figure 7**, P<0.05). These findings demonstrated that ERβ overexpres-

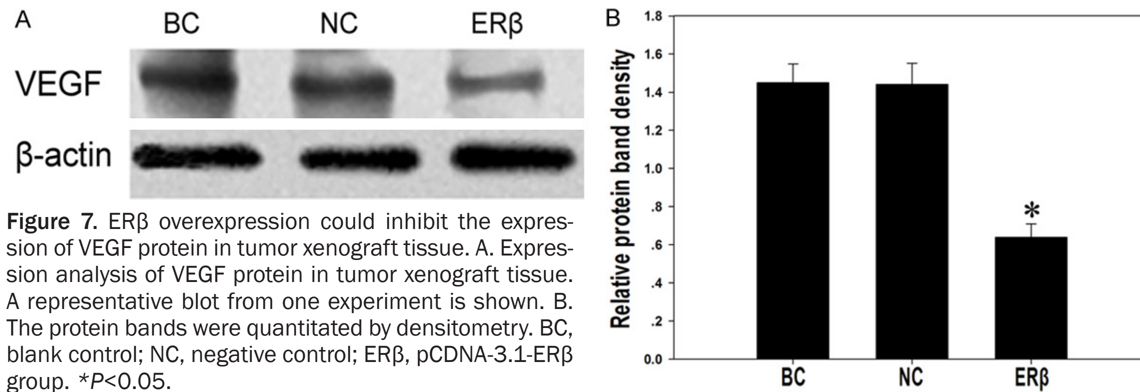

Figure 7. ERβ overexpression could inhibit the expression of VEGF protein in tumor xenograft tissue. A. Expression analysis of VEGF protein in tumor xenograft tissue. A representative blot from one experiment is shown. B. The protein bands were quantitated by densitometry. BC, blank control; NC, negative control; ERβ, pCDNA-3.1-ERβ group. *$P<0.05$.

sion inhibited tumor formation via activating phosphorylation of p38 protein.

Discussion

Prostate cancer (PC) is one of the biggest threats to men's health. During androgen-dependent progression, prostate cancer cells depend on the androgen receptor as the primary mediator of growth and survival. Several studies have shown a decreased expression of ERβ in prostate carcinoma compared to non-pathological tissues [13, 14]. Kim et al have reported that there is positive expression of ERβ in androgen-dependent PC3 cells [15]. ERβ inhibits proliferation and promotes differentiation in the prostate, mammary gland, colon, lung and bone marrow stem cells [16]. The potential beneficial anti-neoplastic role of ERβ in prostate is thus crucial but remains to be demonstrated. In this study, we detected the molecular mechanism of prostate tumorigenesis in androgen-dependent prostate cancer cells with ERβ transfection.

To illustrate the molecular mechanism between ERβ and p38 signal pathway, we tested the expression of p38 and p-p38 proteins, and the biology behavior of LNCaP cells in vitro experiment. The result in **Figure 1** indicated that there was no significant difference of p38 expression between groups. However the expression of p-p38 protein was highest in pCDNA-3.1-ERβ group and lowest in pCDNA-3.1-ERβ+SB203580 group. These results suggested that ERβ overexpression increased the phosphorylation of p38 protein. In order to discuss the effect of overexpression of ERβ on the biological behavior of prostate cancer LNCaP cells, we have successfully established stable ERβ-transfected LNCaP cells. With ERβ overexpression in vivo experiment, the expression of p38 protein was no significant difference among the three groups. However, there was increased ERβ expression and increased p-p38 expression in pCDNA-3.1-ERβ group compared to other groups. All these results confirmed that ERβ overexpression could inhibit the growth of malignant tumors in vivo by repressing the activation of p38.

As ERβ was correlated with prostate cancer, we sought to investigate the role of ERβ in prostate cancer. Previous study has reported that ERβ was overexpressed in human breast adenocarcinoma cell line MCF7 [4, 17]. In the study, we demonstrated that the overexpression of ERβ in LNCaP cells caused a strong inhibition of proliferation. In addition, the result was further confirmed by that the introduction of SB203580 (inhibitor of MAPK pathway) caused a strong promotion of proliferation. These data suggested that ERβ could inhibit cell proliferation by suppressing cell mitosis.

Flow cytometry results showed that ERβ overexpression could increase the number of apoptosis cells, which was inhibited by the inhibitor (SB203580) of MAPK pathway. These result identified that ERβ overexpression could promote cell apoptosis. The result was consistent with previous report that ERβ shRNA increased cell apoptosis [4]. Many proteins are involved in the progress of proliferation and apoptosis. Cyclin D1 is a cell cycle regulator and alters cell cycle progression. Overexpression of Cyclin D1 gene, are observed frequently in a variety of tumors and may contribute to tumorigenesis [18]. The Bcl-2 gene is a proto-oncogene, which encodes the Bcl-2 protein and can inhibit apop-

tosis [19]. Four main Bcl-2 anti-apoptosis mechanisms were followed: antagonistic to the expression of apoptotic Bax gene; inhibiting the release of apoptotic cytochrome c from mitochondria to the cytoplasm; blocking cytoplasmic protein cytochrome c from activating caspase; anti-oxidation and the maintenance of intracellular calcium homeostasis [20-22]. In the study, the expression of cyclin D1 and Bcl-2 protein was lowest in ERβ-transfected group and highest in pCDNA-3.1-ERβ+SB203580 group. The result could be inhibited by inhibitor SB203580. These result suggested that ERβ can inhibit cell proliferation and promote apoptosis by repressing the expression of cyclin D1 and Bcl-2 protein.

The development of tumorigenesis included the invasion and metastasis of tumor. In the study, wound healing assay, soft colony forming assay and transwell invasion assay were used to detect the differentiation and aggressive effect of LNCaP cells. Soft colony forming assay showed that ERβ could inhibit the formation of colony. Wound healing assay demonstrated that ERβ repressed the shift of LNCaP cells. In addition, transwell invasion assay suggested that ERβ decreased the invasion cell number of LNCaP cells. These data proved that ERβ overexpression could inhibit cell differentiation and invasive malignant.

Previous studies have been reported that cell adhesion factors, the hydrolase of extracellular matrix protein and angiogenesis factor could be involved in tumor invasion and formation. Matrix metalloproteinase (MMPs) is extracellular matrix protein lease and break down the histological barrier during tumor cell invasion. MMPs play a key role in the tumor invasion and metastasis [23]. MMP-2 as one of MMPs family acts as a “drill” role in the collagen matrix and the basement membrane between the enzyme in cell [24, 25]. Vascular endothelial growth factor (VEGF) as a key factor in angiogenesis can stimulate endothelial cell proliferation and promote angiogenesis [26]. In the study, we detected the protein expression of MMP-2 and VEGF. The result showed that ERβ overexpression could reduce the expression of MMP-2 protein. In vivo experiment, the protein expression of VEGF gene was also reduced by ERβ overexpression. These data suggested that ERβ could inhibit malignant biological behaviors of LNCaP cell via activating p38 signaling pathway and ERβ overexpression could promote cell differentiation and invasive malignant by repressing the protein expression of MMP-2 and VEGF.

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Disclosure of conflict of interest

None.

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