

## Original Article

# Estrogen receptor $\beta$ inhibits prostate cancer cell proliferation through downregulating TGF- $\beta$ 1/IGF-1 signaling

Long Xiao, Minhui Xiao, Min Zou, Wanchao Xu

Department of Urology, The First People's Hospital of Yunnan Province, Kunming University of Science and Technology, Kunming, Yunnan Province, P. R. China

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**Abstract:** Recently, estrogen receptor  $\beta$  (ER $\beta$ ) appears to be anti-proliferative and pro-apoptotic in normal prostate gland, but its role in androgen independent prostate cancer is limited. In this study, the expression of ER $\beta$  was overexpressed in two androgen-independent prostate cancer cell lines, PC-3 and DU145 after transfection with Ad-ER $\beta$ -EGFP virus particles. Overexpressed ER $\beta$  significantly inhibited cell proliferation in these two prostate cancer cell lines using MTT assay. Flow cytometry and Annexin V-APC/7-AAD double staining confirmed that upregulation of ER $\beta$  increased cell apoptotic rate. We found that upregulation of ER $\beta$  suppressed the expression of TGF- $\beta$ 1/IGF-1 expression, which could be reversed by ER $\beta$ -selective antagonist PHTPP. Consistently, TGF- $\beta$ 1 inhibitor LY2109761 treatment could weaken the effects of ER $\beta$ -selective antagonist PHTPP on the expression of IGF-1, survivin and bcl-2 in prostate cancer cells. In conclusion, these results suggest that estrogen may play an important role in androgen-independent prostate cancer cell proliferation through ER $\beta$ -mediated suppression of TGF- $\beta$ 1/IGF-1.

**Keywords:** Prostate cancer cells, ER $\beta$ , cell proliferation, TGF- $\beta$ 1/IGF-1

## Introduction

Prostate cancer is biologically an extremely heterogeneous disease with high metastasis, and one of the most common male cancers worldwide [1]. There were nearly 1.1 million new prostate cancer cases worldwide and around 307,000 deaths from prostate cancer in the year 2012 [2]. Several pathogenic factors are associated with prostate cancer, including advancing age, ethnicity, inheritance, hormones, diet etc. [3]. Androgen-deprivation therapies of surgical and medical castration are initially effective in the management of prostate cancer, but the majority of tumors will evolve into androgen-independent prostate cancer, however the molecular mechanism of which is not well known [4].

ER $\alpha$  and ER $\beta$  are estrogen receptors belonging to the nuclear receptor superfamily [5]. In addition to ER $\alpha$ , the second estrogen receptor ER $\beta$  was first cloned in the rat prostate, both two were found to play critical roles in modulating

estrogen biological function when bound to ligand [6]. Emerging evidence indicates that estrogen and its receptors are implicate in the tumorigenesis and progression of prostate cancer [7]. The ER $\beta$  expression is diminished or absent in clinically localized and hormone-refractory prostate tumors, it also showed a downward trend with the malignant degree increasing [8]. ER $\beta$  serves as an important function in regulating NF- $\kappa$ B p65 signaling pathway though mediated by HIF-1 in prostate cancer [9]. Zhang *et al.* [10] proposed a pathway control of prostate epithelial cellular proliferation, which composed of ER $\beta$ , 3 $\beta$  Adiol (an ER $\beta$  ligand), and CYP7B1, with ER $\beta$  appears to exert anti-proliferative activity. What's more, ER $\beta$  agonist causes apoptosis in benign prostatic hyperplasia and androgen-independent prostate cancer cells and mediated by TNF $\alpha$  [11].

The cytokines of the transforming growth factor- $\beta$  (TGF- $\beta$ 1) is associated with various cancer types and expressed at higher levels in tissues of prostate and breast carcinomas than

in the normal tissues [11]. It is a dual function regulator that either acts as a tumorigenesis inhibitor in normal prostate gland or tumorigenesis promoter in advanced prostate cancer cells [11]. Recent data showed that TGF- $\beta$ 1 also has an impact on the regeneration of vascular, activation of matrix, and immunodepression [12]. Interestingly, the expression and function of estrogen receptors can be modulated by TGF- $\beta$ 1 in estrogen receptor-positive breast cancer cells [13]. While, the correlation between TGF- $\beta$ 1 and ER $\beta$  in androgen-independent prostate cancer is unknown, they draw our attentions.

Insulin-like growth factors (IGF-1), a growth-promoting polypeptide, provide potent proliferation and survival stimuli in many epithelial tumor cells, including breast cancer cells [14] and prostate cells [15]. Previous study confirmed a potential relationship between TGF- $\beta$ 1 and IGF-1. IGF-1 has been found to be upregulated by TGF- $\beta$ 1 in prostate cancer [11]. The balance between IGF-1 and IGFBP-3 make the TGF- $\beta$ 1 to exert dual biological function on tumorigenesis, migration and growth of prostate cancer [6]. In the present study, androgen-independent prostate cancer PC-3 and DU145 cells were transfected with Ad-ER $\beta$ -EGFP virus particles to overexpress ER $\beta$ . Subsequently, we tried to preliminarily elucidate the effect of ER $\beta$  on prostate cancer cells viability and apoptosis, as well as whether there is an association among ER $\beta$ , TGF- $\beta$ 1, IGF-1 in prostate cancer.

## Materials and methods

### *Cell culture and treatment*

The human androgen independent prostate cancer (PCa) cell lines, PC-3 and DU145 were obtained from the American Type Culture Collection (Manassas, VA, USA). They were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 100 units/ml of penicillin, and 100  $\mu$ g/ml of streptomycin. These two cell lines were maintained in humidified incubators with 5% CO<sub>2</sub> at 37°C. In the following experiments, the cells were also untreated or pretreated with ER $\beta$ -selective antagonist PHTPP (10 nM; Tocris Bioscience) for 30 min. Afterwards, the pretreated cells were incubated in the absence or presence of TGF- $\beta$ 1 inhibitor LY2109761 (10 nM) for 24 h.

### *Cell transfection and treatment*

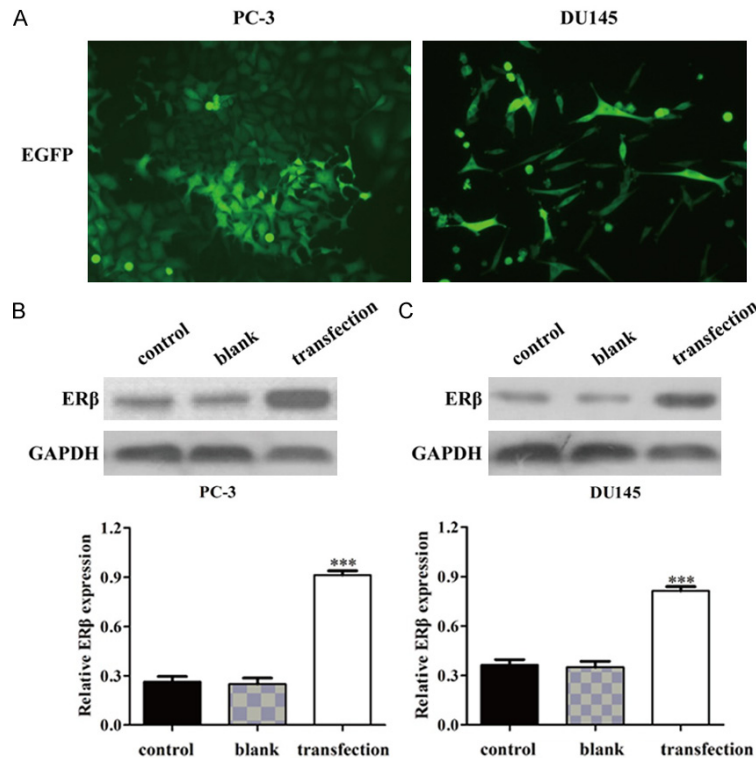
Ad-ER $\beta$ -EGFP virus particles were constructed according to the previous method [16]. PC-3 and DU145 cells were added into a 96-well plate at a density of  $2 \times 10^5$  cell/well and then transfected at a virus particle concentration of  $5 \times 10^8$ /well. The transfection efficiency was evaluated by determining the enhanced green fluorescent protein (EGFP) expression. After transfection, cells were classified as three groups, including un-transfected cells were selected as controls. The cells transfected with Ad-EGFP empty plasmid served as the blank group. The cells transfected with Ad-ER $\beta$ -EGFP served as the transfection group.

### *Real-time quantitative PCR (RT-qPCR)*

The total RNA was isolated from PCa cells using Trizol reagent (Invitrogen) strictly according to the manufacturer's instruction and reverse-transcribed into cDNA using SuperScript III First-Strand Synthesis SuperMix for RT-PCR (Invitrogen). Then the mRNA level of ER $\beta$  was examined by Real-time PCR with the SYBR Green RNA PCR kit (Fermentas, Shenzhen, China) following the manufacturer's instructions. The following primers were used: hER $\beta$ -forward, 5'-CAAGCTCATCTTTGCTCCAGA-3'; hER $\beta$ -reverse, 5'-GCCTTGACACAGAGATATTCTTTG-3'; GAPDH-forward, 5'-CGACCACTTTGTCAAGCTCA-3'; GAPDH-reverse, 5'-AGGGGAGATTCACTGTGGTG-3'. The reactive procedure was shown as follows: 1 min at 95°C, 40 cycles of 95°C for 5 s, and 60°C for 20 s. Gene expression was normalized to the expression of GAPDH by the  $2^{-\Delta\Delta Ct}$  method. Each reaction was repeated at least three times and GAPDH was used as an internal control.

### *Western blotting*

Total proteins were extracted from cells using RIPA lysis buffer (Beyotime, China) and then quantified using Enhanced BCA Protein Assay Kit (Beyotime, China). Then equivalent proteins of each sample were separated by SDS-PAGE on 10% polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore, USA). PBS containing 5% non-fat milk was used to block nonspecific binding for 2 h at room temperature. Subsequently, the membranes were incubated with primary antibodies, includ-



**Figure 1.** Upregulation of ER $\beta$  expression in androgen independent prostate cancer cell lines. (A) Enhanced green fluorescent protein (EGFP) expression was recorded under a fluorescence microscope. The representative images shown are from one of three independent experiments. Western blotting of ER $\beta$  protein levels and RT-qPCR analysis of ER $\beta$  mRNA levels in (B) PC-3 and (C) DU145 cells following transfection. \*\*\* $P < 0.001$  vs control or blank.

ing anti-ER $\beta$ , anti-TGF- $\beta$ 1 and anti-IGF-1 polyclonal antibody IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA; 1:2000) at 4°C overnight. The membranes were rinsed three times with PBS for 5 min and then incubated with the corresponding horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. After washing, the target proteins were visualized by an enhanced chemiluminescence detection system. GAPDH was used as an internal control.

#### MTT assay

For MTT assay, cells were seeded in 96-well plates at a density of  $2 \times 10^3$  cells per well and incubated with 20  $\mu$ L MTT (0.5 mg/ml, Sigma, USA) for 4 h at 37°C. Then each well was added 150  $\mu$ L dimethylsulfoxide (DMSO, Sigma) and incubated for 1 h. Finally, the absorbance of each well at a wavelength of 595 nm was measured for up to 24 h prior to daily analysis on an ELISA reader (Bio-Rad).

#### Apoptosis assay

Cell apoptosis was analyzed by flow cytometry with Annexin V-APC/7-AAD Apoptosis Detection Kit (Key GEN Bio-TECH, Cat no. KGA1026). Briefly, transfected cells were washed twice with PBS, collected in 6-cm dishes at  $4 \times 10^5$  cells/well, and then resuspended in 500  $\mu$ L Annexin-binding buffer. Subsequently, cells were added 5  $\mu$ L of Annexin V-APC and mixed well, then added 5  $\mu$ L of 7-AAD. A flow cytometer BD Biosciences) was used to detect apoptosis and all data was analyzed using Flowjo Software (Treestar).

#### Statistical analysis

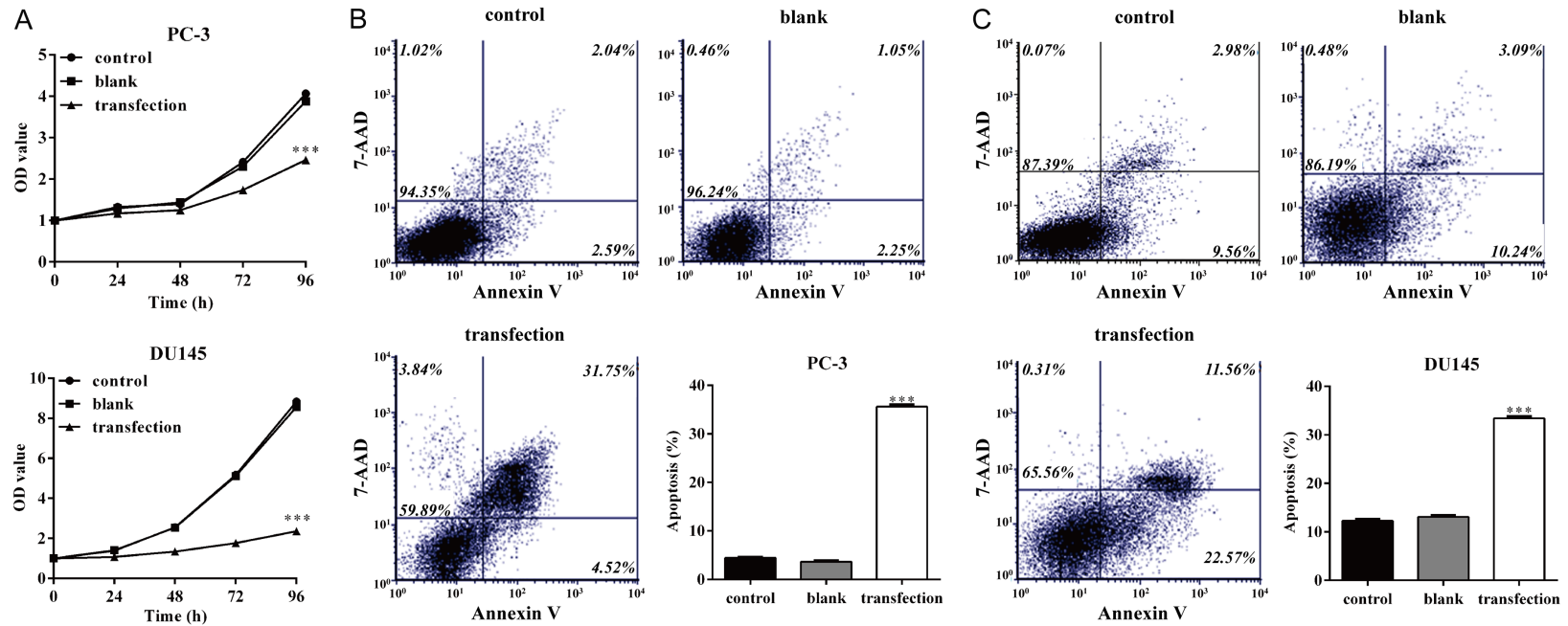
The values are expressed as the mean of at least three different experiments  $\pm$  S.D. The statistical analyses were performed using the SPASS 19.0 software. Student's t-test was used to evaluate the difference between groups and  $P < 0.05$  was considered statistically significant.

## Results

### The expression of ER $\beta$ was upregulated in androgen independent PCa cells

To investigate the role of ER $\beta$  in the development and progression of prostate cancer, the expression of ER $\beta$  was upregulated in two PCa cell lines, PC-3 and DU145 after transfection with Ad-ER $\beta$ -EGFP virus particles. As shown in **Figure 1A**, more than 80% of PC-3 and DU145 cells were EGFP protein positive in transfection groups under fluorescence microscopy, indicating successful infection efficiency. To further evaluate the overexpressed efficiency, we used RT-qPCR and Western blotting to detect ER $\beta$  expression in these two cell lines. As expected, the protein and mRNA levels of ER $\beta$  were significantly upregulated in PC-3 (**Figure 1B**,  $P < 0.001$ ) and DU145 (**Figure 1C**,  $P < 0.001$ ) cells

## Estrogen receptor $\beta$ inhibits prostate cancer cell proliferation



**Figure 2.** Upregulation of ER $\beta$  affected cell proliferation and apoptosis in androgen independent prostate cancer cell lines. (A) MTT assay was used to determine cell proliferation in PC-3 and DU145 cells after cell transfection. Flow cytometry with Annexin V-APC/7-AAD double staining was used to evaluate cell apoptosis in (B) PC-3 and (C) DU145 cells after cell transfection. \*\*\* $P<0.001$  vs control or blank.

after transfection with Ad-ER $\beta$ -EGFP virus particles. The data suggest that constructed Ad-ER $\beta$ -EGFP particles could efficiently enhance the expression of endogenous ER $\beta$  in androgen independent PC-3 and DU145 cells.

### *Upregulation of ER $\beta$ suppressed cell proliferation and promoted cell apoptosis in androgen independent PCa cells*

The effect of ER $\beta$  overexpression on cell proliferation was firstly assessed by MTT assay. As shown in **Figure 2A**, the growth curve of transfected cells started to drop from the second day, compared with blank and control groups. Further analysis indicated that overexpression of ER $\beta$  remarkably decreased OD value from  $3.88 \pm 1.324$  to  $2.46 \pm 0.324$  on day 4<sup>th</sup> in PC-3 cells. Consistently, the OD value was reduced from  $3.51 \pm 0.527$  in blank group to  $2.36 \pm 0.659$  transfection groups on day 4<sup>th</sup> in DU145 cells ( $P < 0.001$ ). Furthermore, we examined whether ER $\beta$  overexpression affected cell apoptotic rate in both PC-3 and DU145 cells. As depicted in **Figure 2B**, the percentage of apoptotic cells was significantly increased from  $4.32\% \pm 0.32\%$  in blank group to  $35.64\% \pm 0.82\%$  in transfection group in PC-3 cells ( $P < 0.001$ ). Similar results were also observed in DU145 cells following transfection (**Figure 2C**,  $P < 0.001$ ).

### *Upregulation of ER $\beta$ could inhibits TGF- $\beta$ 1/IGF-1 signaling in androgen independent PCa cells*

TGF- $\beta$ 1 has been identified as the most potent and universal growth factor that can independently induce mesenchymal transition in various types of cells [17]. But whether its expression was regulated by ER $\beta$  in androgen independent PCa cells remains undefined. We then conducted Western blot analysis to examine the levels of TGF- $\beta$ 1 and IGF-1 in PCa cells under different conditions. As shown in **Figure 3A** and **3B**, upregulation of ER $\beta$  obviously decreased the expression levels of TGF- $\beta$ 1 and IGF-1 in both PC-3 and DU145 cells. Consistently, under the treatment of ER $\beta$ -selective antagonist PHTPP, the expression of TGF- $\beta$ 1 and IGF-1 was remarkably elevated, suggesting that TGF- $\beta$ 1 and IGF-1 are involved in the regulation of androgen independent PCa cell proliferation induced by PHTPP. Moreover, we found the TGF- $\beta$ 1 inhibitor LY2109761 significantly reduced the expression of IGF-1 and survivin in

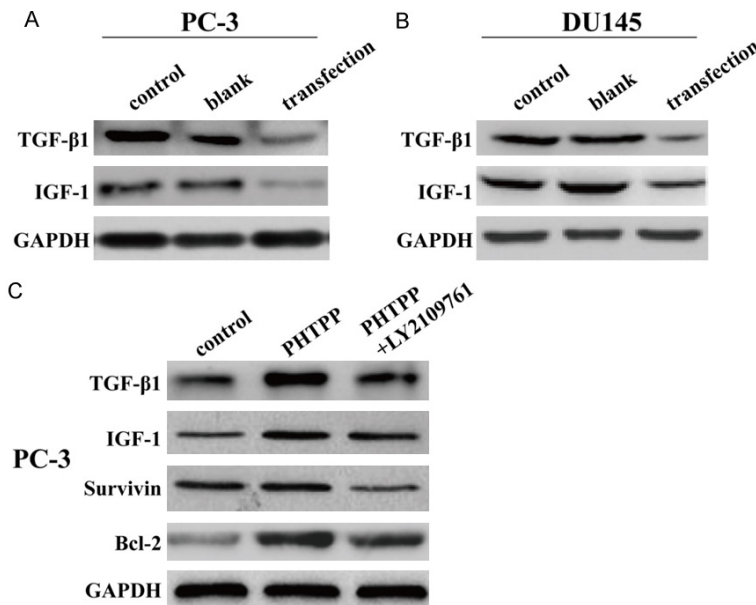
the presence of PHTPP. In addition, bcl-2, as anti-apoptotic marker was increased in the solely presence of PHTPP, but obviously decreased under the LY2109761 treatment. These evidences might indicate that ER $\beta$  affected androgen independent PCa cell proliferation by regulating TGF- $\beta$ 1/IGF-1 signaling.

## Discussion

Prostate cancer is one of the most frequent cancer of the man, particularly in Western country [18]. In most cases, prostate cancers are androgen-dependent and response well to androgen-deprivation therapy, but can be easily switched from androgen dependence to androgen-independence [19]. Estrogen, functions to regulate various processes in numerous tissues, and likely due to the expression profile of a repertoire of genes that controlled by estrogen receptors ER $\alpha$  or ER $\beta$  [20]. The connection among estrogen, tumor promoter, and/or suppressor have been identified, delineating a potential role for estrogen receptor in tumor progression [21]. However, the molecular mechanism of ER $\beta$  in androgen-independent prostate cancer is poorly understood. In this study, transgenic overexpression of ER $\beta$  increased androgen-independent prostate cancer PC-3 and DU145 cells viability, meanwhile allowing the induction of apoptosis in these cells. These results indicate an anti-proliferative activity of ER $\beta$  on androgen-independent prostate cancer, which similar to previous studies as described by McPherson *et al.* [22].

Then, Western blotting was used to determine the effect of ER $\beta$  upregulation on the protein levels of growth factors TGF- $\beta$ 1 and IGF-1, which have been implicated in the development and progression of cancer [23]. As a result, significant downregulation of TGF- $\beta$ 1 and IGF-1 were observed in androgen-independent prostate cancer cells with ER $\beta$  overexpression. Under the treatment of ER $\beta$ -selective antagonist PHTPP, the expression of TGF- $\beta$ 1 and IGF-1 was remarkably reduced, and further decreased in the presence of TGF- $\beta$ 1 kinase inhibitor LY2109761, supporting ER $\beta$  management on TGF- $\beta$ 1 and IGF-1 protein expressions. Meanwhile, IGF-1 might locate in the downstream of TGF- $\beta$ 1 and ER $\beta$ . An alteration of TGF- $\beta$ 1 and IGF-1 levels, indicating that both two are potential positive regulators of growth of androgen-independent prostate cancer cells.





**Figure 3.** Upregulation of ER $\beta$  downregulated TGF- $\beta$ 1/IGF-1 signaling androgen independent prostate cancer cell lines. Effects of ER $\beta$  on TGF- $\beta$ 1 and IGF-1 expression in (A) PC-3 and (B) DU145 cells after transfection using Western blotting analysis; (C) Effects of sole ER $\beta$ -selective antagonist PHTPP or combined PHTPP and TGF- $\beta$ 1 inhibitor LY2109761 on TGF- $\beta$ 1, IGF-1, survivin and bcl-2 in PC-3 cells. GAPDH was used as an internal control.

In addition, **Figure 3C** presents the change of Survivin and Bcl-2 expression patterns with PHTPP alone or mixed with LY2109761, which shows the similar changing tendency to TGF- $\beta$ 1 and IGF-1. We supposed that Survivin and Bcl-2 might act downstream of TGF- $\beta$ 1 in androgen-independent prostate cancer. Survivin is an anti-proliferative/pro-apoptotic protein overexpressed in numerous tumor cell lines [24]. Previous studies unequivocally endorses that the degree of survivin is relate to the prostate cancer progression and development [25]. The protein Bcl-2 is a key regulator of apoptotic, and contributes to cancer cell migration, invasion and metastasis, and aggressiveness of cancer [26].

Recent studies have confirmed that there exist potential associations between TGF- $\beta$ 1 and survivin, as well as Bcl-2. TGF $\beta$  signals were initiated by TGF- $\beta$ 2, then TGF- $\beta$ 1 was recruited to form a TGF- $\beta$ 2/TGF- $\beta$ 1 complex to regulate a series of cellular processes including invasion, cell growth, apoptosis, and tumorigenesis [27]. In prostate epithelial cells, TGF- $\beta$ 1 activity is necessary for the inhibition of survivin by TGF $\beta$  signaling, which downregulates survivin expression though recruitment of Rb and E2F4 to sur-

vivin proximal promoter elements CDE and CHR [27]. Additionally, survivin was found to be upregulated in retinal pigment epithelial cells (ARPE-19) following treatment of TGF $\beta$ 1, when survivin was depleted by siRNA, cell viability was blocked and apoptosis induced by TGF $\beta$ 1 was increased [28]. Motyl *et al.* [29] pointed out that the induction of apoptotic process is associated with a lowered Bcl-2 expression in L1210 leukemic cells when exposed to TGF- $\beta$ 1. Previous study pointed out that the molecular mechanisms of TGF- $\beta$ 1 mediates its inhibitor of human umbilical vein endothelial cells are through decreased expression of bcl-2 [29]. In addition, the up-regulation of Bcl-2 was observed in rat heats with TGF- $\beta$ 1 treatments. These studies

indicate that TGF- $\beta$ 1 may have multifunctional role in positive or negative regulating survivin and Bcl-2. It acts as a tumorigenesis inhibitor in normal prostate gland but function to promote tumorigenesis in advanced prostate cancer cells [30]. In this study, TGF- $\beta$  receptor kinase inhibitor LY2109761 has been shown to decrease survivin and Bcl-2 protein levels, as well as IGF-1. It is likely that TGF- $\beta$ 1 may positively regulate survivin and Bcl-2 proteins expression in androgen-independent prostate cancer cells, thereby contributing to suppression of cell viability and induction of apoptosis.

In addition, there also exist potential associations between IGF-1 and Bcl-2/survivin. Vaira *et al.* [31] demonstrate that the decreased level of survivin in prostate cancer cells is correlated with inhibition of IGF-1/mTOR signaling, accompanied by decreased cell viability. IGF confers the resistance of melanoma cells to apoptosis through promotion of Bcl-2, BCL-X(L) and survivin expression [32]. We supposed that IGF-1 may also regulate the protein expression of survivin and Bcl-2. In conclusion, TGF- $\beta$ 1 and IGF-1 were down-regulated in androgen-independent prostate cancer cells with ER $\beta$  overexpression. The survivin, Bcl-2, TGF- $\beta$ 1 and IGF-1 proteins were

decreased under treatment with PHTPP mixed with LY2109761 compared to PTPPP treatment, which indicates that survivin and Bcl-2 were managed by TGF- $\beta$ 1 and/or IGF-1 in androgen-independent prostate cancer cells.

Further, we conclude that upregulation of ER $\beta$  inhibits androgen-independent prostate cancer cells viability and induces apoptosis possibly through downregulation of anti-apoptotic proteins survivin and Bcl-2 mediated by TGF- $\beta$ 1 and/or IGF-1 signaling pathway. Hence, therapeutic overexpression of ER $\beta$  may be clinically useful to fight against androgen-independent prostate cancer.

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## Disclosure of conflict of interest

None.

**Address correspondence to:** Wanchao Xu, Department of Urology, The First People's Hospital of Yunnan Province, Kunming University of Science and Technology, 157 Jinbi Alley, Kunming 650041, Yunnan Province, P. R. China. Tel: +86-871-6363-8558; Fax: +86-871-63638558; E-mail: xiaoxu-wangchao@163.com

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