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Research Paper

Estrogen receptor β exhibited anti-tumor effects on osteosarcoma cells by regulating integrin, IAP, NF-kB/BCL-2 and PI3K/Akt signal pathway

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ABSTRACT

This study aimed to investigate the effects of Estrogen receptor β (ERβ) on osteosarcoma cells, and explore the regulatory mechanisms involved in this process. Osteosarcoma U2-OS cells consisted four groups, and treated by E2, E2 + LY294002 (ERβ agonists), E2 + ERβ siRNA, E2 + ERβ siRNA + LY294002, respectively. Cell counting kit 8 (CCK-8) assay was performed to detect the cell viability of U2-OS cells in each group. The effects of ERβ on the migration and invasion ability of U2-OS cells were examined by wound healing assay and transwell cell culture chamber, respectively. The expression of Inhibitor of apoptosis protein (IAP) and integrin α5 in U2-OS cells of each group was detected by quantitative RT-PCR, and the expression of phosphorylated p65 (p-p65), p-AKT and Bcl-2 was detected by western blotting. The cell viability, migration and invasion ability of U2-OS cells were significantly increased by ERβ siRNA, but inhibited by ERβ agonists LY294002 ($p < 0.05$). ERβ siRNA significantly downregulated Integrin α5 and unregulated IAP in U2-OS cells ($p < 0.05$). The expression of p-p65, p-AKT and Bcl-2 was significantly reduced by LY294002, but increased by ERβ siRNA ($p < 0.05$). In conclusion, ERβ exhibited obvious anti-tumor effects on osteosarcoma cells by regulating integrin, IAP, NF-kBBCL-2 and PI3K/Akt signal pathway.

1. Introduction

Osteosarcoma is a serious primary malignant bone tumor characterized by the presences of malignant mesenchymal cells and bone stroma [1]. As osteosarcoma usually develops in growing bones, it is most common in teenagers and young adults [2]. In clinic, osteosarcoma exhibits a high rate of propensity for local invasion and early lung metastasis [3]. Despite great advances have been made in the treatment of osteosarcoma, the prognosis of patients remains poor. According to statistics, the 5-year survival of localized osteosarcoma was about 65–70%, while it is only 20% in metastatic osteosarcoma [4]. Therefore, identifying novel therapeutic targets and further understanding the mechanisms involved in tumorigenesis of osteosarcoma are urgently needed.

Estrogen receptor β (ERβ), firstly discovered in 1996, is a hormone-regulated transcription factor, which exerts its effects on target tissues by interacting with estrogen [5]. Since ERβ expression was identified to be decreased during tumor progression by various researches, ERβ has been considered as a potential therapeutic target in tumors. It has been reported that ERβ was an important modulator in the inhibition of proliferation, invasion, and tumor formation of MCF-7 breast cancer cells [6]. Estrogen-activated ERβ was a tumor suppressor, which could inhibit the cell proliferation, migration, invasion, and increase the apoptosis of renal cell carcinoma cells [7]. ERβ agonists Liquiritigenin and LY500307 could significantly inhibit the growth and promote the apoptosis of glioblastoma in vivo [8]. Estrogen is important in bone growth during puberty to bone remodeling in adult, while related researches on the regulatory role of ERβ on osteosarcoma are still limited.

The mechanisms underlying ERβ-mediated anti-tumor response are complex, and various factors are revealed to be involved in this process. It has been reported that ERβ could inhibit colon cancer xenograft growth through inhibiting cell-cycle pathways (p21, p27 and p45) [9]. The tumor repressive function of ERβ in human malignant pleural mesothelioma was interrelated with epidermal growth factor receptor (EGFR) inactivation [10]. ERβ was contributed to the suppression of breast cancer cells through regulating multiple components of transforming growth factor β (TGFβ) signaling pathway [11]. However, the regulatory mechanisms of ERβ on osteosarcoma have not been fully explained, and a deeper research is still needed.

U2-OS cell lines (originally 2T), firstly isolated from moderately

Abbreviations: ERβ, Estrogen receptor β; EGFR, epidermal growth factor receptor; TGFβ, transforming growth factor β; NC, control; FBS, fetal bovine serum; CCK-8, Cell counting kit 8; OD, optical density;; PAGE, SDS-polyacrylamide gel electrophoresis

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differentiated osteosarcoma do not have any adenoviral infections, and specifically no large T antigen. Due to the characteristics of fast growth and high transfection efficiency, U2-OS cell lines are considered as an ideal osteosarcoma cell line in the researches of ER. In this study, the effects of ERβ on osteosarcoma cells, including cell viability, migration and invasion ability were evaluated by using ERβ agonists LY294002 and ERβ siRNA. Meanwhile, the expression of IAP, integrin α5, p-p65, p-AKT and Bcl-2 was detected to reveal the regulatory mechanisms of ERβ on U2-OS cells. Our finding may provide a novel theoretical basis for clinical treatment of osteosarcoma in future.

2. Materials and methods

2.1. Cell culture and treatment

Human osteosarcoma cell line U2-OS (purchased from Shanghai Tongpai Biological Technology Co., Ltd., China) was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (GIBCO, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) at 37 °C with 5% CO_2. The medium was refreshed every 72 h. Logarithmic growth phase cells (80–90% confluence) were used for the following treatments.

The sequences of ERβ siRNA (5′-AAGCCCAAATGTGTTGTGGCC-3′) and control (NC) (5′-TTCTCCGAACGTGTCACGTTT-3′) were synthesized in Shanghai GenePharma Co., Ltd., China. U2-OS cells were divided into four groups: NC + E2, NC-transfected U2-OS cells treated by 10^{-10} M E2; NC + E2 + LY294002, NC + E2 + 45 μM LY294002 (Sigma, Louis, MO, USA); ERβ siRNA + E2, ERβ siRNA-transfected U2-OS cells treated by 10^{-10} M E2; and ERβ siRNA + E2 + LY294002, ERβ siRNA + E2 + 45 μM LY294002.

2.2. Cell viability assay

Cell counting kit 8 (CCK-8) was performed to detect the cell viability of U2-OS cells in different groups according to the instructions (Beyotime, Nanjing, China). Simply, U2-OS cells of each group were seeded at a density of 0.5×10^4/well in 96-well plates. After 0, 24, 36 and 48 h of treatments, 10 μg CCK-8 (Sigma) was added into each plate. The optical density (OD) at 450 nm was detected by a Microplate reader (Thermo Scientific, Waltham, MA, USA).

2.3. Cell migration assay

The effect of ERβ on migration of U2-OS cells was examined by wound healing assay. After 0, 24, 36 and 48 h of the above treatments, U2-OS cells were seeded at a density of 0.5×10^6/well in 6-well plates, and then cultured overnight (90% confluence). A wound track was scored in each dish with a pipette head. The debris was removed by washing with PBS. After 0, 24 and 48 h of culturing, the migration distances were visualized and photographed (Olympus IX71, Japan).

2.4. Cell invasion assays

The invasion assay was performed to evaluate the effect of ERβ on the invasion ability of U2-OS cells using transwell cell culture chambers (Beyotime). The U2-OS cells were firstly digested with Trypsin (Beyotime), washed with PBS, and then suspended in RPMI-1640 serum-free medium containing 0.2% BSA. A total of 200 μL U2-OS cells at a density of 0.1×10^7/mL were added to the upper compartment of the chamber (pre-coated with Matrigel), and 600 μL FBS were placed in the lower compartment of the chemotaxis chamber. After incubation at 37 °C for 24 h, cells on the upper surface of the filter were removed with cotton swabs, and those on the lower side were fixed in formaldehyde for 30 min and then stained with 0.1% crystal violet for 20 min. Stained cells were observed through an inverted microscope (Olympus IX71, Japan). More than 3 fields of views at 200× magnifications were counted randomly.

2.5. Quantitative RT-PCR

Quantitative RT-PCR was performed to detect the expression of ERβ, IAP and integrin α5. Total RNA of U2-OS cells of each group was isolated and reversed transcribed using special kits (Universal RNA Extraction Kit, PrimeScript® RT reagent Kit with gDNA Eraser, Takara, Dalian, China). The mRNA expression of these genes was detected by SYBR Premix Ex Taq (Takara) on ABI7500 (ABI, USA) using specific primers (ERβ-F: 5′-TGGAACTGGAGAGCTTGGC-3′, ERβ-R: 5′-AGGCACTCCAGAGCAGAAC-3′; IAP-F: 5′-GGCCCTCTTCTGATTTCAGGTT-3′, IAP-R: 5′-TTTGAATGCATT AAGGGGTTCCTC-3′; integrin α5-F: 5′-GGACAGATGCCACACAAGGA-3′, integrin α5-R: 5′-AGGCACTCCAGAGCAGAAC-3′). The PCR program included 95 °C for 1 min, 40 cycles at 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. Relative expression of these genes was normalized to β-actin (β-actin-F: CATCGTCCACCGCAAATGCTTC, β-actin-R: AACCGACTGCTGTCA CCTTCAC).

2.6. Western blotting

U2-OS cells of each group were lysated by RIPA Cell Lysis Buffer (Solarbio, Beijing, China). The total proteins were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on 10% polyacrylamide gels, and transferred to a polyvinylidenefluoride membrane (Millipore, Billerica, MA, USA). The membrane was blocked with 5% skim milk in Tris buffered saline Tween (TBST) for 2 h, and then incubated with special primary antibody (anti-p-p65, anti-p-AKT, anti-Bcl-2, 1:2000, Affinity BioReagents, Golden, CO, USA) at 4 °C overnight. β-actin (1:5000, Abcam, Cambridge, MA, USA) was considered as control. After washed with TBST for three times, HRP-conjugated secondary antibody (1:5000, Beyotime) was added and kept on incubation for 2 h at 25 °C. The protein bands were visualized using luminescence buffer (Millipore), and quantified by an UV gel imager (Shanghai Tianneng, Shanghai, China).

2.7. Statistical analyses

Each experiment was performed in triplicate, and all data were expressed as mean ± SD. Statistical analysis was performed by SPSS version 17.0 (SPSS Inc., Chicago, IL). Comparison between different groups was determined by Student's *t*-test (two groups) and one-way ANOVA (more than two groups). A p-value less than 0.05 was considered to be significantly different.

3. Results

3.1. Knockdown of ERβ increased the cell viability of U2-OS cells

To evaluate the regulatory role of ERβ in U2-OS cells, an ERβ siRNA was constructed. As shown in Fig. 1A, the expression of ERβ was significantly reduced in U2-OS cells transfected with ERβ siRNA when compared with that in the control (NC vs. siRNA, $p < 0.01$). The knockdown efficiency of ERβ siRNA was about 70%. Then, the effects of ERβ on the cell viability of U2-OS cells were evaluated. After 24 h of treatment, a significantly lower cell viability was exhibited on U2-OS cells treated by LY294002 than that in the control (NC + E2 vs. NC + E2 + LY294002, $p < 0.05$). However, ERβ siRNA could significantly increase the cell viability of U2-OS cells (NC + E2 vs. ERβ siRNA + E2, $p < 0.01$). Meanwhile, the cell viability of U2-OS cells treated by both LY294002 and ERβ siRNA was significantly higher and lower than that only treated by LY294002 and ERβ siRNA, respectively (NC + E2 + LY294002, ERβ siRNA + E2 vs. ERβ siRNA + E2 + LY294002, $p < 0.05$). With the extension of treatment times, similar variation tendency of cell viability was revealed on 36 and 48 h post-treatment (Fig. 1B).

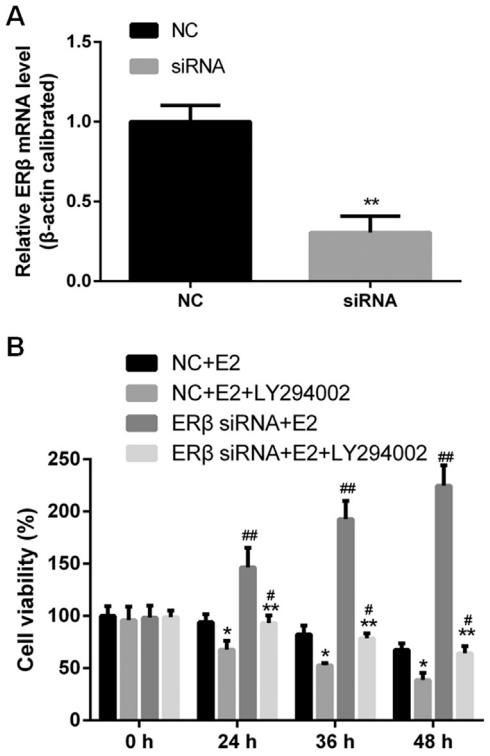


Fig. 1. A) The expression of Estrogen receptor β (ERβ) in U2-OS cells treated by ERβ siRNA. **, $p < 0.01$, control (NC) vs. ERβ siRNA (siRNA). B) The cell viability of U2-OS cells treated by LY294002 or/and ERβ siRNA in the presence of 10^{-10} M E2. *, $p < 0.05$, **, $p < 0.01$, NC/ERβ siRNA + E2 vs. NC/ERβ siRNA + E2 + LY294002; ##, $p < 0.01$, #, $p < 0.05$, NC + E2/ NC + E2 + LY294002 vs. ERβ siRNA + E2/ERβ siRNA + E2 + LY294002.

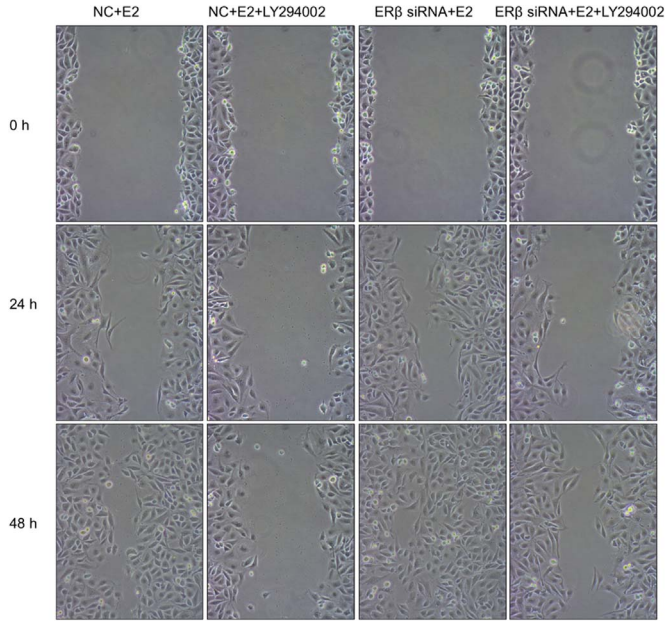


Fig. 2. The cell migration of U2-OS cells treated by LY294002 or/and Estrogen receptor β (ERβ) siRNA in the presence of 10^{-10} M E2 (200 X).

3.2. Knockdown of ERβ promoted the cell migration of U2-OS cells

By the treatment of LY294002 for 24 h, the migration distance was significantly reduced in U2-OS cells. A significantly increased migration distance was exhibited on U2-OS cells treated by ERβ siRNA. When compared with U2-OS cells treated by both LY294002 and ERβ siRNA, the migration distance was significantly higher and lower in U2-OS cells only treated by LY294002 and ERβ siRNA, respectively. Meanwhile, the variation tendency of migration distances was more obvious on 48 h post-treatment (Fig. 2).

3.3. Knockdown of ERβ promoted the cell invasion of U2-OS cells

The effect of ERβ on the cell invasion of U2-OS cells was also evaluated. As shown in Fig. 3, the invasion ability of U2-OS cells was significantly reduced by LY294002 (NC + E2 vs. NC + E2 + LY294002, $p < 0.05$), but increased by ERβ siRNA (NC + E2 vs. ERβ siRNA + E2, $p < 0.05$). When compared with U2-OS cells treated by both LY294002 and ERβ siRNA, the invasion ability was significantly higher and lower in U2-OS cells only treated by LY294002 and ERβ siRNA, respectively (NC + E2 + LY294002, ERβ siRNA + E2 vs. ERβ siRNA + E2 + LY294002, $p < 0.05$) (Fig. 3).

3.4. Knockdown of ERβ downregulated Integrin α5 level and unregulated IAP level in U2-OS cells

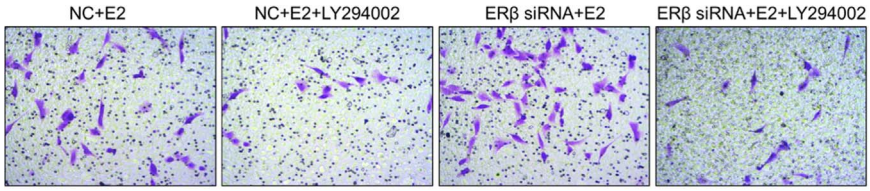
To reveal the regulatory mechanisms of ERβ in U2-OS cells, the expression of integrin α5 and IAP was detected. After 48 h of treatment, LY294002 was revealed to be able to upregulate the expression of integrin α5, while downregulate the expression of IAP (NC + E2 vs. NC + E2 + LY294002, $p < 0.01$). In contrast, significantly reduced integrin α5 and increased IAP was revealed on U2-OS cells treated by ERβ siRNA (NC + E2 vs. ERβ siRNA + E2, $p < 0.01$). When compared with U2-OS cells treated by both LY294002 and ERβ siRNA, the expression of integrin α5 was significantly lower and higher in U2-OS cells only treated by LY294002 and ERβ siRNA, respectively (NC + E2 + LY294002, ERβ siRNA + E2 vs. ERβ siRNA + E2 + LY294002, $p < 0.01$). The expression trend of IAP was just opposite to integrin α5 (NC + E2 + LY294002, ERβ siRNA + E2 vs. ERβ siRNA + E2 + LY294002, $p < 0.01$) (Fig. 4).

3.5. Knockdown of ERβ upregulated p-p65, p-AKT and Bcl-2 in U2-OS cells

The expression of p-p65, p-AKT and Bcl-2 was further detected to explore the regulatory mechanisms of ERβ in U2-OS cells. After 48 h of treatment, the expression of p-p65, p-AKT and Bcl-2 was significantly lower and higher in U2-OS cells treated by LY294002 and ERβ siRNA, respectively (NC + E2 vs. NC + E2 + LY294002, ERβ siRNA + E2, $p < 0.01$). Meanwhile, U2-OS cells treated by both LY294002 and ERβ siRNA exhibited significantly higher and lower expression of p-p65, p-AKT and Bcl-2 than those only treated by LY294002 and ERβ siRNA, respectively (NC + E2 + LY294002, ERβ siRNA + E2 vs. ERβ siRNA + E2 + LY294002, $p < 0.01$) (Fig. 5).

4. Discussion

Recently, ERβ, a traditional estrogen receptor, was revealed to be closely related with the occurrence and development of tumors. The decreased expression of ERβ has been observed in various cancers, such as breast cancer, prostatic cancer, lung cancer and colorectal cancer [12]. What is important, the anti-tumor effects of ERβ have also been reported in osteosarcoma. It has been reported that estrogen inhibitor fulvestrant exhibited obvious anticancer activity on osteosarcoma at high concentrations though downregulating the expression of ERβ [13]. Estrogen could inhibit etoposide-induced apoptosis of human osteosarcoma cells via mediating ERβ [14]. In this study, the cell viability, migration and invasion ability of U2-OS cells were significantly inhibited by ERβ agonists LY294002. Meanwhile, ERβ siRNA significantly increased the cell viability, migration and invasion ability of U2-OS cells. These findings were consistent with previous studies, and further illustrated the anti-tumor role of ERβ on osteosarcoma cells in the



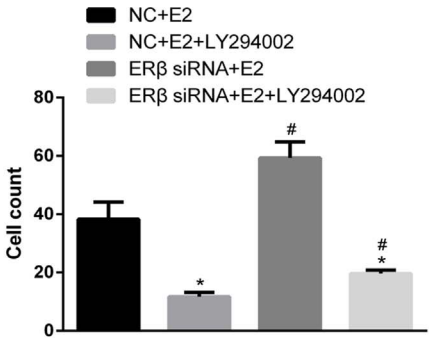


Fig. 3. The cell invasion of U2-OS cells treated by LY294002 or/and Estrogen receptor β (ERβ) siRNA in the presence of 10^{-10} M E2 (200 X). *, $p < 0.05$, NC/ERβ siRNA + E2 vs. NC/ERβ siRNA + E2 + LY294002; #, $p < 0.05$. NC + E2/ NC + E2 + LY294002 vs. ERβ siRNA + E2/ERβ siRNA + E2 + LY294002.

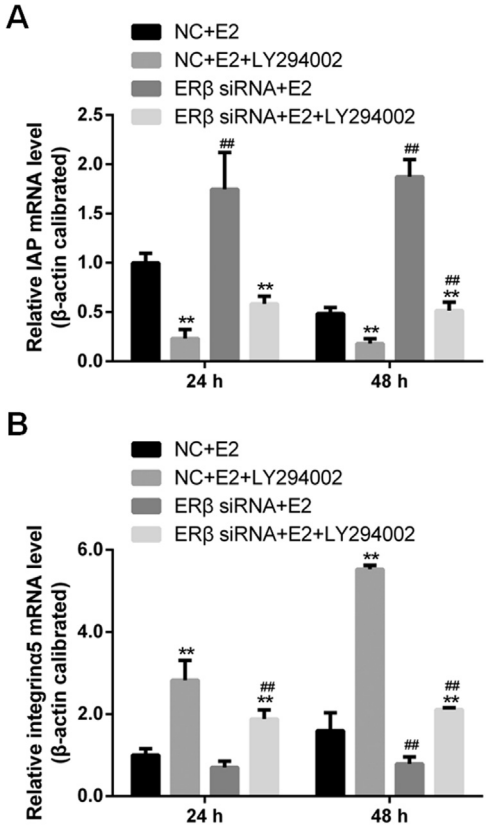


Fig. 4. The expression of IAP (A) and integrin α5 (B) (mRNA level) in U2-OS cells treated by LY294002 or/and Estrogen receptor β (ERβ) siRNA in the presence of 10^{-10} M E2. **, $p < 0.01$, NC/ERβ siRNA + E2 vs. NC/ERβ siRNA + E2 + LY294002; ##, $p < 0.01$. NC + E2/ NC + E2 + LY294002 vs. ERβ siRNA + E2/ERβ siRNA + E2 + LY294002.

presence of E2. ERβ agonists might become novel potential candidates for endocrine therapy in osteosarcoma.

Although the anti-tumor role of ERβ has been identified by evidence points, the related mechanisms still remain elusive [12]. Integrin was a kind of transmembrane receptor, which could promote the adhesion of facilitate cell-extracellular matrix. Since integrin could mediate various cellular signals, it played important roles in complex biological events such as differentiation, development, and tumor progression and aggressiveness [15]. In this study, the expression of integrin α5 was significantly increased by LY294002 and reduced by ERβ siRNA in U2-OS cells. This phenomenon indicated that the anti-tumor role of ERβ was related with increased integrin α5. However, integrin α5 was reported to be related with increased capability of invasion and migration in osteosarcoma MG64 cells [16]. Meanwhile, integrin α5 mediated intravasation could increase tumor metastasis and decrease animal survival of osteosarcoma [17]. This difference may be attributed to the presence of E2. On the other hand, we also found the knockdown of ERβ could upregulate IAP in U2-OS cells. IAP was a kind of protein function in prevention of cell death [18]. The improperly regulated IAP was frequently accompanied with cancer. It has been reported that ViscumTT treatment resulted in synergistic apoptosis induction in osteosarcoma cells by down-regulating IAP [19]. Doxorubicin and Cisplatin sensitized U2-OS osteosarcoma cells to TRAIL by down-modulating IAP family proteins [20]. Downregulation of XIAP could decrease cell proliferation and colony formation, and induce cell apoptosis in osteosarcoma [20]. Therefore, we suspected that the upregulated IAP may also contribute to the anti-tumor effects of ERβ on osteosarcoma.

NF-kB was known as a pro-inflammatory cytokine involved in various biological processes including cell proliferation, differentiation, apoptosis and immune response [21]. Since NF-kB could directly regulate the expression of BCL-2 transcriptionally, NF-kB/BCL-2 pathway was considered to be important in carcinogenesis and apoptosis of tumors [22]. Meanwhile, NF-kB/BCL-2 pathway was also identified to be related to ER in tumors. It has been reported, ER mediated expression of p65 was correlated with invasion and metastasis of HCC [23]. An inverse correlation between ERβ and p65 immunoreactivity was observed in urinary bladder carcinogenesis [24]. ERβ ligands could promote autophagy of hormone-resistant breast cancer cells by reducing Bcl-2 expression [25]. Endometriosis-associated tumors were associated with overexpression of Bcl-2 and reduced expression of ER [26]. To consistent with these researches, we found that the expression of p-p65 and Bcl-2 was significantly reduced in U2-OS cells by LY294002 and increased by ERβ siRNA. Our findings further illustrated that NF-kB/BCL-2 pathway was one of the most important mechanisms involved in ERβ-mediated anti-tumor response on osteosarcoma.

PI3K-Akt signal pathway was an important intracellular signaling pathway in regulation of cell cycle [20]. The abnormal PI3K/Akt signal pathway was frequently observed in human cancers [20]. In this study, the expression of p-AKT was found to be significantly inhibited by LY294002 in U2-OS cells, which was consistent with previous studies [20]. It has been reported, ERβ could independently predict a better prognosis of three negative breast cancer by interacting PI3K/pAKT pathway [27]. The inhibition effects of calycosin on ER-positive breast cancer cells were mediated by PI3K/pAKT pathway [28]. Estrogen

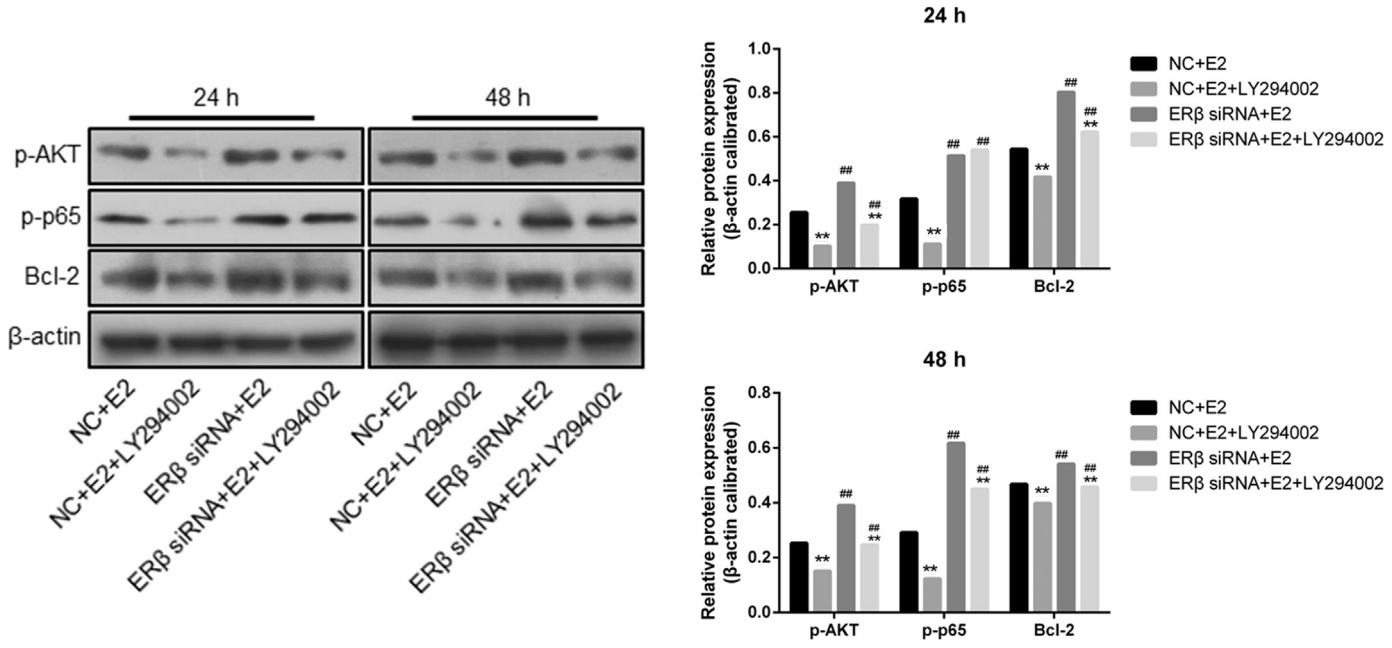


Fig. 5. The expression of p-p65, p-AKT and Bcl-2 (protein level) in U2-OS cells treated by LY294002 or/and Estrogen receptor β (ERβ) siRNA in the presence of 10–10 M E2. **, $p < 0.01$, NC/ERβ siRNA + E2 vs. NC/ERβ siRNA + E2 + LY294002; ##, $p < 0.01$. NC + E2/ NC + E2 + LY294002 vs. ERβ siRNA + E2/ERβ siRNA + E2 + LY294002.

could activate PI3K-Akt pathway through ERβ in breast cancer, and pAkt positivity was associated with poor disease-free survival of patients [29]. Our findings further illustrated that the anti-tumor effects of ERβ were related with PI3K/Akt signal pathway.

5. Conclusion

In conclusion, ERβ exhibited obvious anti-tumor effects on osteosarcoma, which could inhibit the cell viability, migration and invasion ability of U2-OS cells. ERβ-mediated anti-tumor response was closely related with integrin, IAP, NF-kB/BCL-2 and PI3K/Akt signal pathways. However, the related mechanisms have not been fully revealed, and the clinical application of ERβ in the treatment of osteosarcoma was limited. Further researches on these fields were still needed.

Competing interests

The authors declare that they have no competing interests.

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