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Estrogen receptor β signaling regulates the progression of Chinese non-small cell lung cancer

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ABSTRACT

Prospective studies have found that the risk of non-small cell lung cancer (NSCLC) has close relationship with estrogen. The effects of estrogens are mediated via two estrogen receptor (ER) isoforms, that is, ER alpha (ERα) and ER beta (ERβ). ERα in NSCLC has been evaluated mostly by immunohistochemistry. However, our previous study showed that ERβ was also highly expressed in Chinese NSCLC. But the roles of ERβ in Chinese NSCLC have not been clarified as yet. So in the present study, two Chinese lung adenocarcinoma cell lines, SPC-A1 and LTEP-a2, were used and the role of ERβ in lung tumorigenesis was focused to be investigated by *in vitro* and *in vivo* experiments. The results showed that over-expressed ERβ can promote the development of NSCLC, while siRNAs targeting ERβ gene can inhibit growth of NSCLC cells and induce apoptosis of these cells via mitochondrial depolarization and caspase-3 activation. These results indicated that ERβ plays an important role in development of Chinese NSCLC. This suggests that ERβ deactivation or down-regulation may possess potential therapeutic utility for the treatment of lung cancer.

1. Introduction

Lung cancer, especially non-small cell lung cancer (NSCLC), remains the leading cause of cancer-related mortality for both men and women worldwide [1]. A causal relationship between smoking and lung cancer is well established, but differences in smoking patterns are not sufficient to explain apparent biological differences between genders in lung cancer incidence [2]. Women are much more likely than men to be diagnosed with lung adenocarcinoma, while squamous cell carcinoma is more common in men. Gender disparities in adenocarcinoma, which accounts for 75% of lung cancer in females, implicate sexual hormones in lung cancer. Women have naturally higher circulating estrogen levels than men that may increase their susceptibility to lung cancer [3]. Estrogen levels are found to be elevated in female lung cancer patients as compared with those in women without lung cancer. Estrogens may also affect lung cancer growth clinically as full estrogen blocked reduces the occurrence of primary lung cancers in women with breast cancer [4]. In addition, it was reported that relatively high aromatase levels correlated with a worse prognosis of lung cancer outcome. Aromatase is a key enzyme in estrogen biosynthesis and converts the androgens androstenedione and testosterone to estrone and estradiol, respectively [5].

Estrogen signaling pathways may play an important role in development of lung cancer, especially adenocarcinoma [6,7]. The cellular response to estrogen is mediated by estrogen receptorα (ERα) and ERβ. However, it was reported that ERα is not the primary mediator of transcriptional responses to estrogen in NSCLC cells, whereas ERβ seems to be the dominant form in normal lung and lung tumor cell lines and tissues [8], and estrogen-dependent responses in NSCLC cells are probably generated via ERβ [9,10]. ERβ was also reported to be necessary for the maintenance of the extracellular matrix composition in lung, with loss of ERβ leading to abnormal lung structure and systemic hypoxia [11]. Recent study showed that ERβ was the predominant isotype expressed in lung cancer cells. Endogenous ERβ was localized in cytoplasm of NSCLC cells and was unable to translocate to nucleus after estrogen addition [12]. It was also found that ERβ was localized in both cytoplasm and nucleus [13]. Moreover, when expression of either ERα or ERβ was suppressed by small interfering RNA (siRNA), the proliferation of NSCLC cell (NCI-H23, A549) *in vitro* was significantly reduced [14], while Stabile et al. found that ERα was elevated in tumors but was not predictive of survival [15].

Our previous study showed that ERβ was highly expressed in Chinese NSCLC and mainly localized to the nuclei of the cancer cells [16]. However, the roles of ERβ in Chinese NSCLC have not

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been clarified. Thus, to elucidate the action of ERβ in Chinese NSCLC, SPC-A1 and LTEP-a2 were chosen. These two cell lines are all derived from Chinese male patients with lung adenocarcinoma. SPC-A1 has no endogenous expression of ERα and ERβ, while LTEP-a2 possesses high endogenous ERβ and low endogenous ERα. Through construction ERβ-expression plasmid and siRNA technology *in vitro* and *in vivo*, we found that ERβ exerted multiple effects on human lung carcinoma cells both *in vivo* and *in vitro*, and human ERβ specific short interference RNA had anticancer activity.

2. Materials and methods

2.1. Plasmids construction and design of siRNA

Based on information about the ERβ mRNA (AB006590), ERβ eukaryotic expression plasmid were constructed. In our previous work, we have designed pMD18-T-ERβ vector, the human ERβ DNA fragment were prepared by PCR on pMD18-T-ERβ vector respectively using ERβ primers (restriction sites for NheI and BamHI were underlined) listed in Table 1. Then, the product of PCR digested with NheI and BamHI were inserted into the eukaryotic expression plasmid vector pCDNA3.1 (+) (Invitrogen, Carlsbad, CA, USA), which resulted in the formation of pCDNA3.1/ERβ. The resultant recombinant plasmids were characterized by detailed restriction digestion (Fig. 1). Specific sequence for human ERβ Stealth™ Select RNA was designed and synthesized by Ivitrogen (Ivitrogen, Life Technologies, USA). And two suitable sequence was selected: siERβ (sense: 5′ CCACCAUGAAUAUCCAGCCAUGACA 3′, antisense: 5′ UGUCAUGGCUGGAUAUUCAUGGUGGCU3′) and siERβ′ (sense: 5′ UGUCAUGGCUGGAUAUUCAUGGUGG3′, antisense: 5′CCACCAUGCCUAUCAGCCAUGACA3′). Oligo Stealth ™ RNAi Negative Control Duplexes, which is designed to minimize sequence homology to any known vertebrate transcript, were obtained from Invitrogen.

2.2. Cell culture and transfection

Two lung adenocarcinoma cell lines, SPC-A1 and LTEP-a2 (SIBCB, China), were cultured in RPMI medium 1640 (Gibco/ BRL) supplemented with 10% fetal calf serum (PAA, Austria) at 37 °C under 5% humidified CO_2 and 100 μg/ml each of streptomycin and penicillin G (Amresco). The plasmid and siRNA were transfected by Lipofectamine 2000 reagent (Invitrogen, USA) according to the manufacturer's instructions. Briefly, approximately 0.8×10^5 SPC-A1 cells/well were grown overnight in 24-wells plate. When the cells reached 90–95% confluence, they were transfected with 0.8 μg of pCDNA3.1/ERβ or pCDNA3.1 respectively in serum-free medium using Lipofectamine 2000. Approximately $3–4 \times 10^4$ LTEP-a2 cells/well were grown overnight in 24-wells plate. When cells reached to 30–50% confluence, they were transfected with anti-

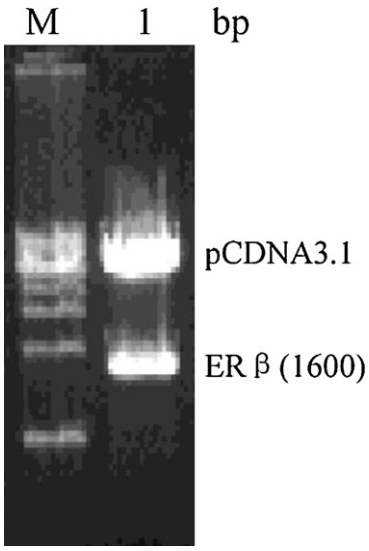

Fig. 1. Restriction digestions of recombinant plasmids. M: 1 kbp DNA Ladder Marker(1000–10000 bp) (TaKaRa); lane 1: pCDNA3.1/ERβ.

ERβ siRNAs (100 nM) and negative control siRNA (100 nM) in serum-free medium using Lipofectamine 2000. After incubation for 4 h at 37 °C, 400 μl RPMI 1640 with 10% FBS was added. Stable transfectants were selected in the presence of 400 μg/ml G418 (Amersco) during 4 weeks of culture. In addition, the concentration of estradiol-β (E2) used in subsequent experiments was 10 nM. Before E2 treatment, the cells were placed in phenol red-free media supplemented with 5% dextran-coated charcoal stripped fetal bovine serum (DCC-FBS) for 72 h.

2.3. RT-PCR and real-time PCR

Total RNA was extracted using the TaKaRa RNAiso Reagent (TaKaRa, Japan) according to the manufacturer's instructions. RNA concentration was quantified by spectrophotometer at 260 nm. 1 μg total RNA was reverse-transcribed using Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas). Subsequently, the mRNA expression of ERβ was determined by RT-PCR. 2 μl of the incubation mixture was used as the template for the following PCR using 2 × Taq Enzyme Mix kit (TianGen, China). In addition, bcl2 and bax mRNA expression were assayed by real-time PCR.SYBR Green qPCR SuperMix-UDG was purchased from Invitrogen (Shanghai, China) and ABI 7300 real-time PCR machine was used. The level of expression was calculated based on the PCR cycle number (Ct), and the relative gene expression level was determined using ΔΔCt method as described [17]. And Oligonucleotides as primers were synthesized by Invitrogen and listed in Table 1.

Table 1
Primers used in this study.

Primers	Sequence5′–3′	Excepted size (bp)	Annealing temperature
f-ERβ sense	CTAGCTAGCATGGATATAAAAAACTCACCATCTAGC	1593	51 °C
f-ERβ antisense	CGCGGATCCCTGAGACTGTGGGTTCTGGGAG		
t-ERβ sense	TCCAGCCATGACATTCTA	180	46 °C
t-ERβ antisense	GAGGTTCCGCATACAGAT		
β-actin sense	TCTGGCACCACACCTTCTA	270	46 °C
β-actin antisense	AGGCATACAGGGACAGCAC		
Bcl-2 sense	AGCTGCACCTGACGCCCTTCACCGC	288	55 °C
Bcl-2 antisense	AGGAGAAATCAAACAGAG		
Bax sense	TGCTTCAGGGTTTCATCCAGG	295	55 °C
Bax antisense	GCCTTGAGCACCAGTTTG		

Restriction sites for *NheI* and *BamHI* are underlined.

2.4. Western blot analysis

1×10^7 cells were lysed in a buffer containing 50 mM Tris–Cl pH8.0, 150 mM NaCl, 0.02% NaN_3, 0.1% SDS, 100 μg/ml phenylmethylsufonyl fluoride (PMSF), 1 μg/ml Aprotinin, 1% Triton. After centrifugation, cell lysates (75 μg/lane) were subjected to 12% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Millipore). The membranes were blocked for 1 h in PBST (10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 0.05% Tween-20) containing 2% nonfat dried milk, and then antibodies against ERβ (Millpore, 1:2000), β-actin (Sigma, 1:400) and HRP-conjugated goat anti-rabbit and anti-mouse secondary antibodies (Promab, 1:1000) were used. Protein bands were detected by the enhanced chemiluminescence (ECL) reaction (Kibbutz Beit Haemek, Israel).

2.5. Cell viability analysis

Cell viability was assessed with a Cell Counting Kit (Dojin Laboratories, Kumamoto, Japan). Briefly, different treated groups of SPC-A1 and LTEP-a2 cells were plated in 96-well plates in RPMI 1640 supplemented with 10% FBS at a density of 3×10^3 cells/well. After 6 h, the medium was changed to serum-free medium, and the cells were cultured ≤2 days. 10 μl of a solution containing WST-8 (4-[3-(2-methoxy-4-nitrophenyl)-2-(4-nitrophenyl) – 2H-5-tetrazolio]-1, 3-benzene disulfonate sodium salt) was added to each well. Following incubation of an additional 4 h, the absorbance was measured at 450 nm with a muti-detection microplate reader (Hynergy™ HT, BIO-TEK).

2.6. Proliferation analysis

Cell proliferation was assessed by CFSE (5,6-carboxyfluorescein diacetatesuccinimidyl ester), which is a kind of fluorescence dye. After labeling the cells, its fluorescence intensity decreased with each cell division. Mean fluorescence intensity, named geometric mean (GEO), can be used as an index to analyze the cell proliferation ability. Less fluorescence intensity manifests the more cell division. Briefly, SPC-A1 cells transfected with pCDNA3.1/ ERβ or pCDNA3.1 and parental SPC-A1 cells were digested by enzyme. These cells were resuspensed with RPMI medium 1640 and CFSE was added at the final concentration of 5 μmol/L. After this mixture was incubated at 37 °C/5% CO_2 for 10 min, medium was poured and cells were cleaned at least twice with PBS. The cells were plated in 24-well plates in RPMI 1640 supplemented with 10% FBS at a density of 1×10^4 cells/well and cultured 2 days. At last, the cells labeled with CFSE were detected by flow cytometry (FCM).

2.7. Annexin V/PI staining

Cells were centrifuged to remove the medium, washed once with binding buffer (10 mM Hepes, 140 mM NaCl, 2.5 mM $CaCl_2$ in aquadest.) and stained with 5 μl Annexin V-FITC at RT for 15 min. 10 μl 20 μg/ml PI was added at RT for 10 min and cells analyzed by flow cytometry. Viable cells were negative for both PI and annexinV; apoptotic cells were positive for annexinV and negative for PI, whereas late apoptotic dead cells displayed both high annexin V and PI labeling. Non-viable cells which underwent necrosis were positive for PI and negative for annexin V.

2.8. Confocal microscopy

NSCLC cells were grown on coverslips in phenol red-free media. Cells were fixed with methanol/acetone and stained with ERβ antibody (H-150, Santa Cruz, CA). Fluorescent images were collected with a confocal scanning laser system (Olympus Fluoview 1000; Olympus Corp., Lake Success,NY) attached to an inverted microscope (IX81; Olympus Corp., Tokyo, Japan).

2.9. Cytochrome c releasing apoptosis assay

Cytochrome c was assayed according to the instruction of Cell Cytochrome c Flow Cytometry Kit (Genmed Scientifics Inc. USA). Briefly, after treatment with or without transfection, 1.5×10^6 cells were collected by centrifugation at $400 \times g$ for 5 min and then treated these cells with different reagents of the kit. Each tube of cell was stained with cytochrome c antibody and its second antibody for 45 min, respectively. At last, the expression of cytochrome c was detected by FCM.

2.10. Determination of the mitochondrial membrane potential by Rhodamine 123

Rhodamine 123 uptake by mitochondria is directly proportional to its membrane potential. LTEP-a2 cells subjected to 72 h after transfection were incubated with Rh 123 (5 μg/ml concentration) for 30 min in dark at 37 °C. The cells were harvested and suspended in PBS. The mitochondrial membrane potential was measured by the fluorescence intensity (FL-1, 530 nm) of 10,000 cells on flow cytometer [18].

2.11. Determination of active caspase-3, -8 and -9

In order to determine the potential role of the caspase- 3, -8 and -9 proteases in the pathways of ERβ knockdown – induced apoptosis, the activities of caspase- 3, -8 and -9 were measured by caspase- 3, -8 and -9 Colorimetric Assay Kit (KeyGen, China) respectively. Briefly, 1×10^6 cells subjected to 72 h after transfection were lysed at 4 °C for 30 min and the supernatant were transferred to a clean microfuge tube, protein concentration was assayed. Then, 50 μl 2× reaction buffer and 5 μl caspase-3, 8, 9 substrate were added, incubated at 37 °C for 4 h in dark. The absorbance was measured at 405 nm on a muti-detection microplate reader (Hynergy™ HT, BIO-TEK). The activity of caspase-3, -8, and -9 was determined by calculating the ratio of OD405 nm of transfected cells/OD 405 nm of parental cells following the instruction of the manufacturer.

2.12. Colony formation in soft agar

To perform the soft agar assay, a base layer of 0.5% (w/v) agar was prepared by adding autoclaved 1% (w/v) agar solution to 2 × RPMI medium 1640 supplemented with 20% fetal calf serum in a 1:1 ratio. 2×10^3 SPC-A1 cells or LTEP-a-2 cells transfected with plasmid or siRNA were prepared in a 1:1 mixture of 0.7% (w/v) agar solution and 2 × RPMI medium 1640 supplemented with 20% FCS. Cell suspension was added to the top of the base layer, allowed to solidify, and incubated at 37 °C/5% CO_2 for 10–15 days. The number of colonies was determined by direct counting under a microscope. Counts are expressed as number of colonies per plate. Average of counts is from three plates per group in triple experiments.

2.13. Migration assay

The migration assay was performed using 24-well transwell chambers (8 μm, Millipore, Billerica, MA). The transfected SPC-A1 cells with pCDNA3.1/ ERβ or pCDNA3.1 and parental cell were suspended in phenol red-free 1640 medium without serum and 2×10^4 cells were seeded onto Matrigel inserts in triplicate. They were then put into a 24-well culture plate containing 500 μl phenol red-free 1640 medium with or without E2. After culture for 12 h, cells on the upper side of the filters were removed with cotton-tipped swabs, and the filters were washed with PBS. Cells on the

underside of the filters were examined and counted under a microscope. Images were taken with four fields randomly selected from each insert. The number of cells in each field was counted and averaged. Migration is expressed as fold increase over control.

2.14. Tumorigencity study in vivo

Female BALB/c nude mice (5–6 weeks old) were obtained from Experimental Animal Limited Company of Vitalriver of Peking. Cultured cells were harvested by trypsinization, washed and suspended in PBS at 10^7 cells/ml. 100 μl cell suspension was injected subcutaneously into the flank of female nude mice (seven mice per cell line). Tumor diameters were monitored at 14, 21 and 28 days, and tumor volume in mm^3 was calculated by the formula: volume = $(\text{width})^2 \times \text{length}/2$. Data were presented as means ± SE. 28 days after injection, nude mice were sacrificed, and the tumors were removed, photoed and weighed. All procedures were approved by Institutional Animal Care and Use Committee of Nanjing University.

2.15. Immunohistochemistry

Immunostaining was performed on 6 μm tissue sections using strept–avidin–biotin staining kit (Boster, China). For antigen retrieval, slides were heated by microwave in 0.01 M Tri–Sodium Citrate buffer. Nonspecific binding sites were blocked with 5% BSA for 30 min and endogenous peroxidase activity was suppressed by treating with 3% H_2O_2 in methanol for 30 min. Sections were exposed to rabbit polyclonal anti- ERβ and ant-ERα antibody (Ready-to-use, spring) overnight at 4 °C. The chromogen was 3,3-diamino-enzidine (Boster). Counterstaining was done with hematoxylin. Negative control sections were incubated with PBS instead of anti-ERβ antibody. In each step, samples were carefully washed with PBS.

2.16. Statistical analysis

Results were presented as mean ± S.E.M. Statistical significance between groups was analyzed by one-way ANOVA followed with the Student–Newman–Keuls multiple comparisons tests. A p-value of <0.05 was considered significant. Frequency of tumorigenesis in nude mice was calculated by Fisher's exact test.

3. Results

3.1. Construction of ERβ over-expressed SPC-A1 cell line and siRNA targeting ERβ transfection down-regulates the corresponding mRNA and protein levels in LTEP-a2 cells

To evaluate the role of ERβ in non-small cell lung tumorigenesis, we used transfection assay to obtain an ERβ over-expressed lung cell line and used siRNAs to downregulate the ERβ expression in LTEP-a2. RT-PCR and Western blotting were performed to determine ERβ expression level in the transfected lung adenocarcinoma cells. Compared with the parent cells and pCDNA3.1 transfected cells, transfection of SPC-A1 cells with pCDNA3.1/ERβ resulted in significantly elevating ERβ expression in SPC-A1 cells (Fig. 2A and B). When LTEP-a2 cells were transfected with the siERβ and siERβ′, ERβ mRNA (Fig. 2C) and proteins (Fig. 2D) were down-regulated 72 h later, but negative control siRNA did not affect the ERβ mRNA and protein. These results indicated that transfection of pCDNA3.1/ERβ and anti-ERβ siRNAs were successful.

3.2. Effects of over-expressed ERβ on SPC-A1 cells and down-regulated ERβ on LTEP-a2 in vitro

To determine whether the change of expression of ERβ can affect the non-small cell lung cancer cells proliferation, cell proliferation activity was detected using Cell Counting Kit. The transfection of pCDNA3.1/ ERβ to SPC-A1 significantly increased the proliferation of SPC-A1 cells while the transfection of anti-ERβ siRNAs to LTEP-a2 significantly decreased the proliferation of LTEP-a2 compared with the control. This showed that up-expressed and down-expressed ERβ can accordingly accelerate and inhibit the cellular proliferation at 24, 36 and 48 h, respectively. No difference between SPC-A1/vector and SPC-A1, either between LTEP-a2/si Control and LTEP-a2 were detected (Fig. 3A and B). SPC-A1 cell proliferation activity and LTEP-a2 cell apoptosis were also determined by flow cytometry. Fig. 3C and D showed that less fluorescence (GEO mean) was released from SPC-A1/ERβ cells than from SPC-A1/vector and SPC-A1 cells. It indicates that ERβ can promote SPC-A1 cell division. After knocking out ERβ with siRNAs, the apoptosis cells increased (Fig. 3E). These results were similar to those by Cell Counting Kit. Furthermore, to test potential malignant state of the tumor cell line, colony-forming assay was conducted in different treatment groups of SPC-A1 (Fig. 3F) and LTEP-a2 (Fig. 3G). The results showed that the colony numbers of SPC-A1/ ERβ and LTEP-a2/siERβ cells were accordingly increased and inhibited by 2- to 3-fold compared with control cells. In addition, the results in migration assay also showed that over-expressed ERβ could induce significantly migration of the cell at 12 h, about 3.5- fold over SPC-A1 and SPC-A1/vector cells (Fig. 3H). Furthermore, the state of SPC-A1 was irrelevant to presence or absence of E2 (Fig. 3). These results demonstrated that the changes in ERβ expression could significantly alter the malignant state and migration ability of non-small cell lung cancer.

3.3. ERβ was localized in nucleus of LTEP-a2 and both nucleus and cytoplasm of SPC-A1/ ERβ

ERβ has genomic or non-genomic function according its location. The result of confocal assay showed that ERβ was localized in nucleus of LTEP-a2 (Fig. 4). After transfection, ERβ was localized both in nucleus and cytoplasm of SPC-A1 (Fig. 4).

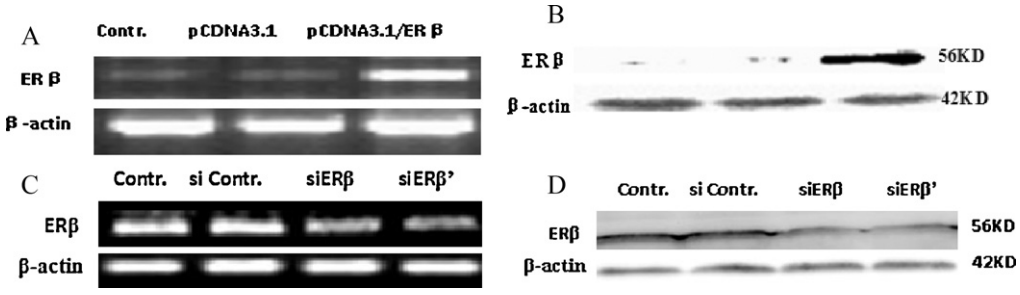

Fig. 2. RT-PCR and Western blotting analysis of the expression of ERβ in SPC-A1 and LTEP-a2 after transfection. (A) ERβ gene level in SPC-A1 after transfection; (B) ERβ protein level in SPC-A1 after transfection; (C) ERβ gene level in LTEP-a2 after transfection; (D) ERβ protein level in LTEP-a2 after transfection.

3.4. Inhibition of ERβ expression decreased mitochondrial membrane potential

The loss of mitochondrial membrane potential is a hallmark for apoptosis. It is an early event coinciding with caspases activation. The effect of ERβ siRNA on the mitochondrial transmembrane potential was measured using rhodamine 123. The increase of percentages of apoptotic ERβ siRNA transfected cells was observed (Fig. 5A). Apoptotic cells in parental LTEP-a2 and nagtive siRNA transfected LTEP-a-2 cells were approximately 3.29%, 3.3%. However, the rate of apoptotic ERβ siRNA transfected cells increased to 9.46% in LTEP-a2 (Fig. 5B).

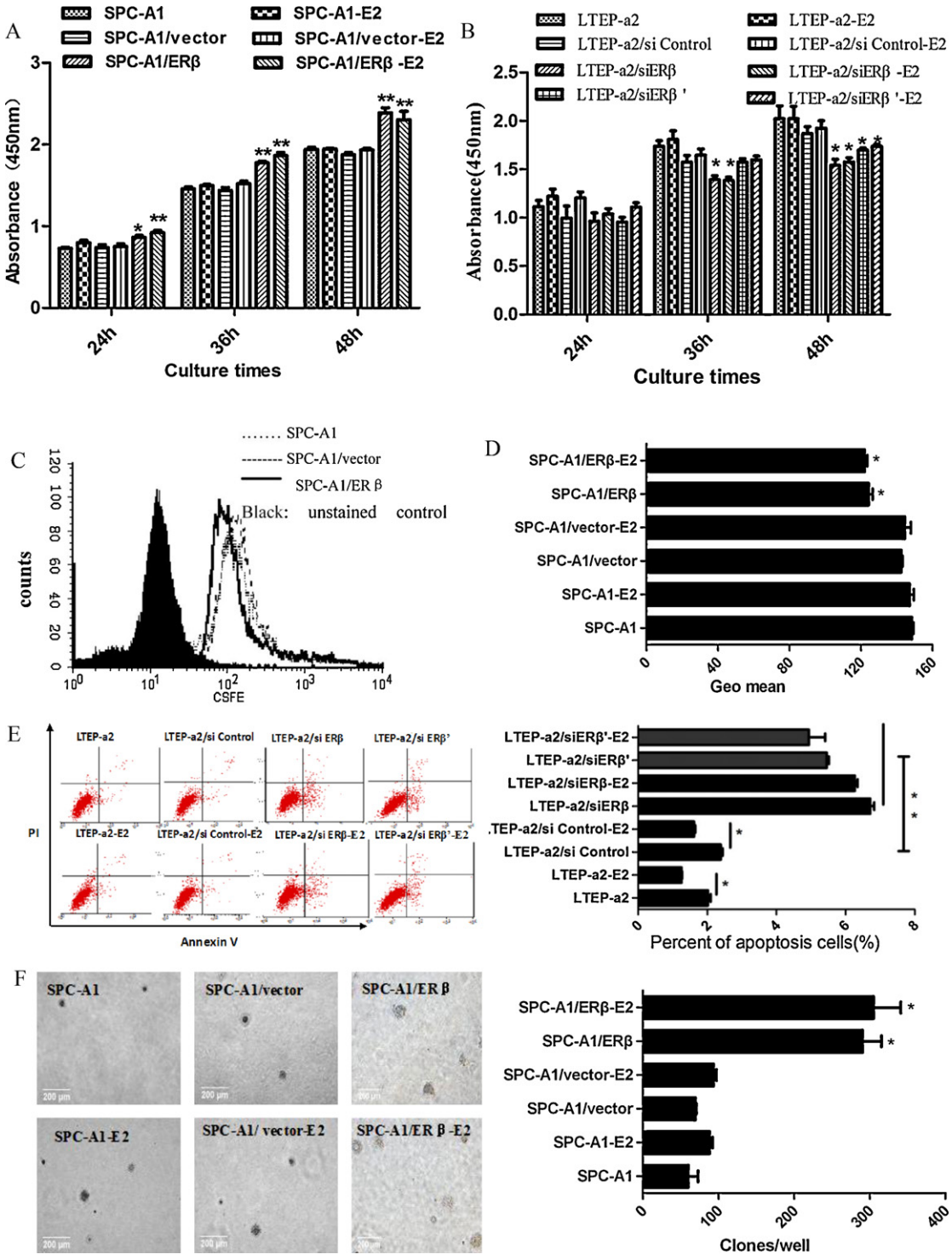

Fig. 3. Change of ERβ expression affects SPC-A1 and LTEP-a2 cell proliferation, cell apoptosis, colony formation and cell migration *in vitro*. (A) SPC-A1 cell proliferation determined by Cell Counting Kit; (B) LTEP-a2 cell proliferation determined by Cell Counting Kit; (C) SPC-A1 cell proliferation determined by flow cytometry and overlay of cell division; (D) Geo mean statistics. Less fluorescence intensity manifests the more cell division; (E) Effects of Anti-ERβ siRNAs on LTEP-a2 cells discriminated by Annexin-V/PI double stain; (F) SPC-A1 colony formation in soft agar observed under light microscope and counted the colony numbers. (G) LTEP-a2 colony formation in soft agar observed under light microscope and counted the colony numbers. (H) Analysis of cell migration. (I) SRC-1 protein level in SPC-A1 and LTEP-a2 after transfection. - E2 stands for estradiol-β treatment group. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

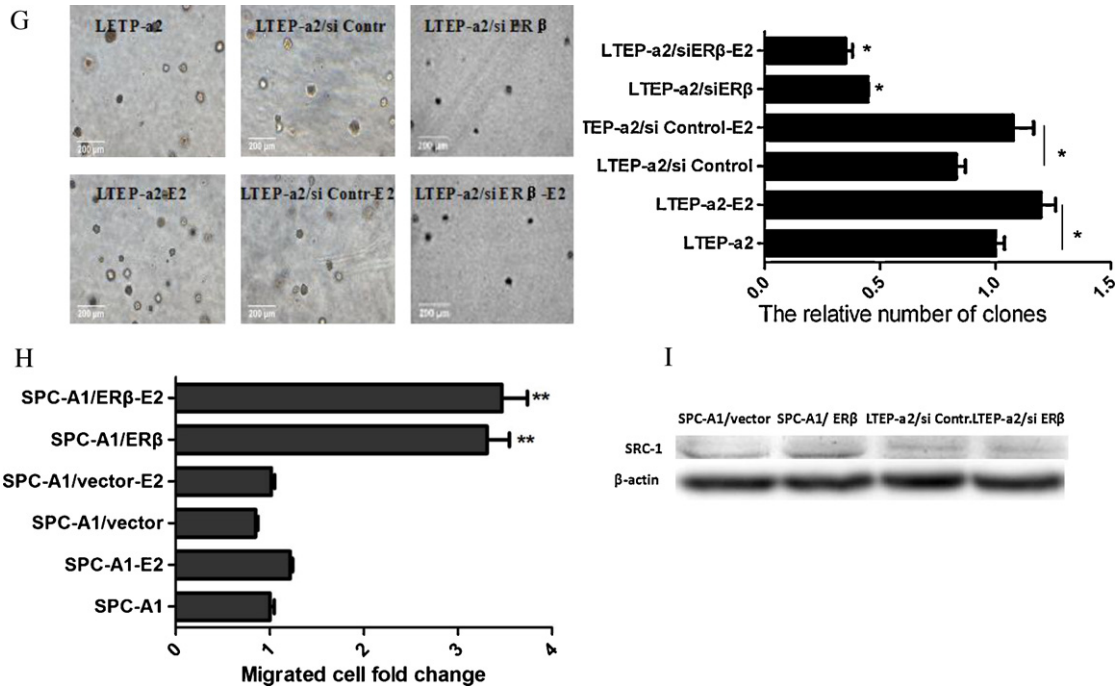

Fig. 3. (*Continued*).

3.5. Inhibition of ERβ expression down-regulated expression of bcl-2 and up-regulated expression of bax and release of cytochrome c

Bcl-2 family proteins have a central role in controlling mitochondria apoptosome-mediated apoptotic pathway; the Bcl-2/Bcl-Xl subfamily proteins inhibit apoptosis, while the Bax/Bak subfamily promotes cell death [19]. In this study, we detected the bcl-2 and bax expression level by real time-PCR after siRNA target ERβ transfection (Fig. 6). The mRNA expression of bcl-2 was decreased in LTEP-a2 after siRNA target ERβ transfection, while bax was increased (Fig. 6A).

Fig. 6B shows that after LTEP-a2 cells transfected with anti-ERβ siRNA, the release of cytochrome c from the mitochondria into cytosol was increased, compared with parental cells and cells transfected with negative RNA.

3.6. Activation of caspase-3 and -9 via inhibition of ERβ expression

The caspase cascade is activated during apoptosis. Therefore, we examined the activity of caspase-3, 8 and 9 in LTEP-a2 transfected with ERβ siRNA or with negative RNA (Fig. 7) by colorimetric assay. In addition to executioner caspase-3, initiator caspase-8 and -9 are also important for apoptosis. The ratio of OD405 nm of transfected cells/ OD 405 nm of parental cells was calculated. In LTEP-a2 cells, caspase- 3 and -9 changed significantly.

3.7. Tumor growth promoted by ERβ in vivo

Over-expressed ERβ significantly changed the behavior of SPC-A1 cells *in vitro*, so it is necessary to analyze the tumorigenicity of the above stable transfectant cells *in vivo*. Tumor diameters and

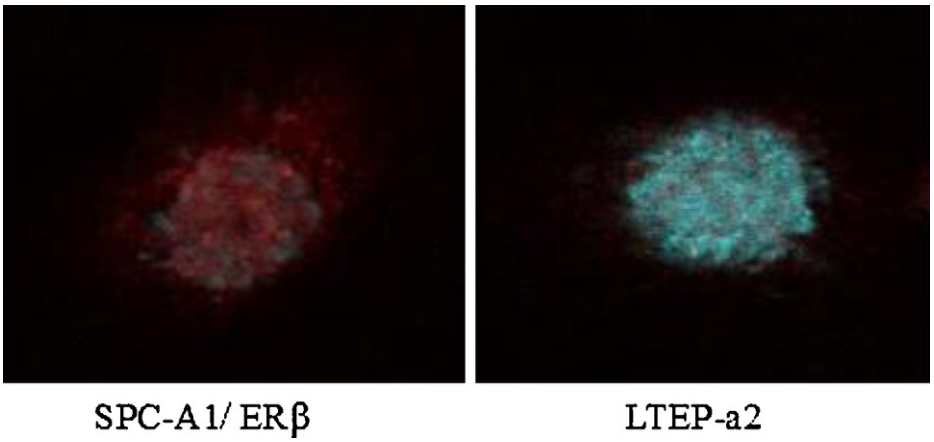

Fig. 4. ERβ location analysis. SPC-A1/ERβ and LTEP-a2 were grown on coverslips. Then confocal microscopy studies were performed with ERβ antibody. ERβ (red) and nucleus (blue) staining are merged. (×200). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

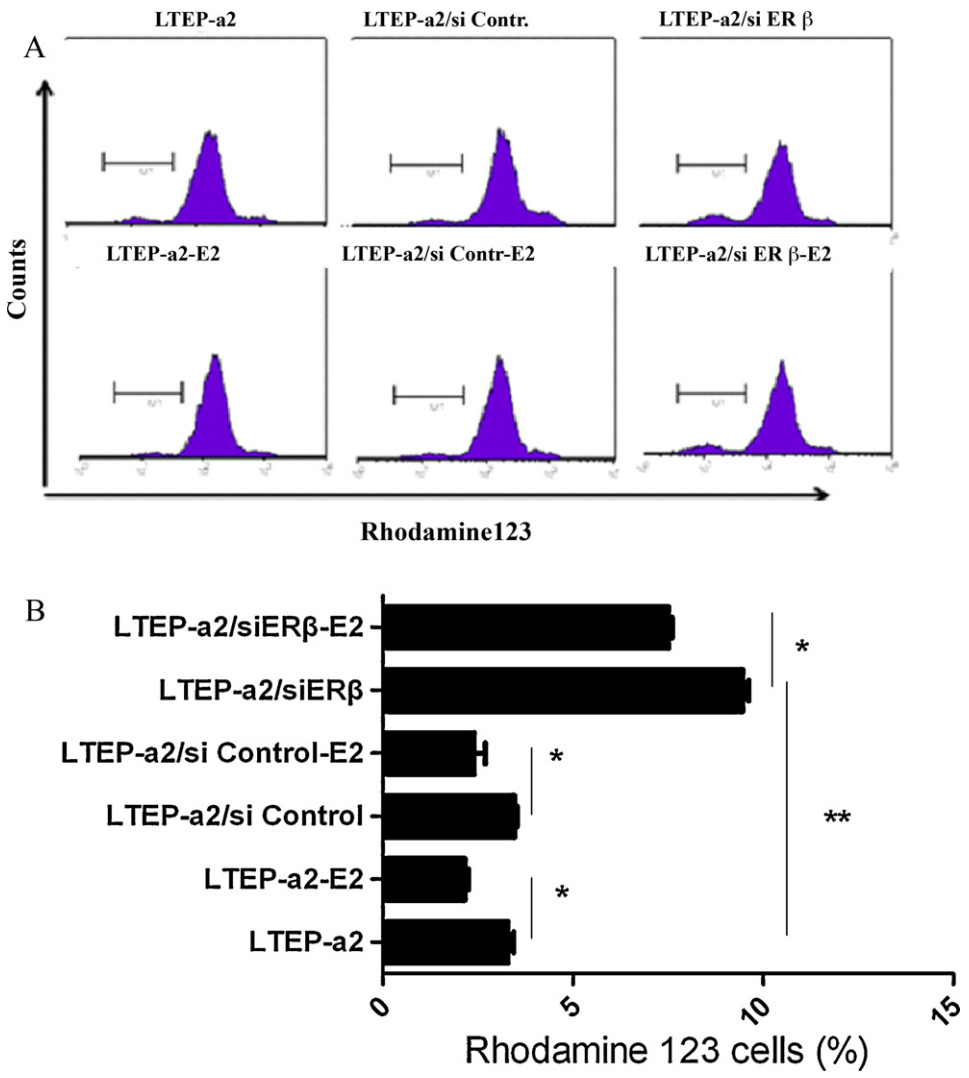

Fig. 5. Changes in the mitochondrial transmembrane potential detected with rhodamine 123. (A) The reduced fluorescence of rhodamine 123 was determined as the reduced mitochondrial transmembrane potential in histogram. Data are representative of three experiments. (B) Graph shows the percentages of Rhodamine123 positive cells which represent the mean ± S.E.M from three independent experiments. $^{**}p < 0.01$ vs. control.

volume in mm^3 were subsequently measured at 28 days. At day 28 after injection, the tumors were removed and photoed. Apparently, SPC-A1/ ERβ- transfected cells could multiply and grow more early and rapidly than the control SPC-A1 and SPC-A1/vector cells in nude mice (Fig. 8A). Tumor volumes of mice injected with SPC-A1/ ERβ cells were significantly larger than those of the control at day 28 (Fig. 8B). Tumor growth rate (TGR) from 21 to 28 day were evaluated, the results showed that the TGR of nude mice injected with SPC-A1/ ERβ was significantly rapider than that of control cell injected mice (Fig. 8C).

3.8. Immunohistochemical analysis

To detect whether SPC-A1/ ERβ transfected cells can stably express ERβ in nude mice for a long period as well as the association of tumor growth with ERβ protein levels, immunohistochemical staining was used. The specific brown-yellow color was identified as positive results. We observed that ERβ was expressed in cytoplasm and nucleus of tumor cells from different treatment group of mice. The number and density of the positive points in tumor tissue induced with SPC-A1/ ERβ cells were evidently higher than those of tumor tissue with SPC-A1 and SPC-A1/vector cells (Fig. 9).

4. Discussion

Prospective studies have found that the risk of NSCLC has close relationship with estrogen [20,21]. Our previous results showed that ERβ was over-expressed in Chinese NSCLC and its positive protein expression rate was exclusively related to the pathological classification in NSCLC [16]. In order to explain if the expression of ERβ affects the development of Chinese NSCLC, we selected two cell lines which deprived from Chinese lung adenocarcinoma patients. Constructed ERβ-expression plasmid and selected ERβ specific siRNAs were successfully transfected into SPC-A1 and LTEP-a2, respectively (Fig. 2).

ERβ expression affects NSCLC cells. Cell counting kit was used in this study for analysis of cell viability. The results showed that over-expressed ERβ enhanced the growth of SPC-A1 cells, while ERβ siRNAs inhibited LTEP-a2 cells growth. However, the cell growth in E2 treatment group had no significant difference from that in control group. In order to determine the effects of E2 on NSCLC, CFSE and Annexin V-PI staining assay was used. The CFSE results showed that over-expressed ERβ indeed promoted the proliferation of SPC-A1 cells, while E2 still had no effect on proliferation of SPC-A1 cells (Fig. 3C). 1 nM and 100 nM E2 were also used to confirm this phenomenon (data not shown). These results were inconsistent

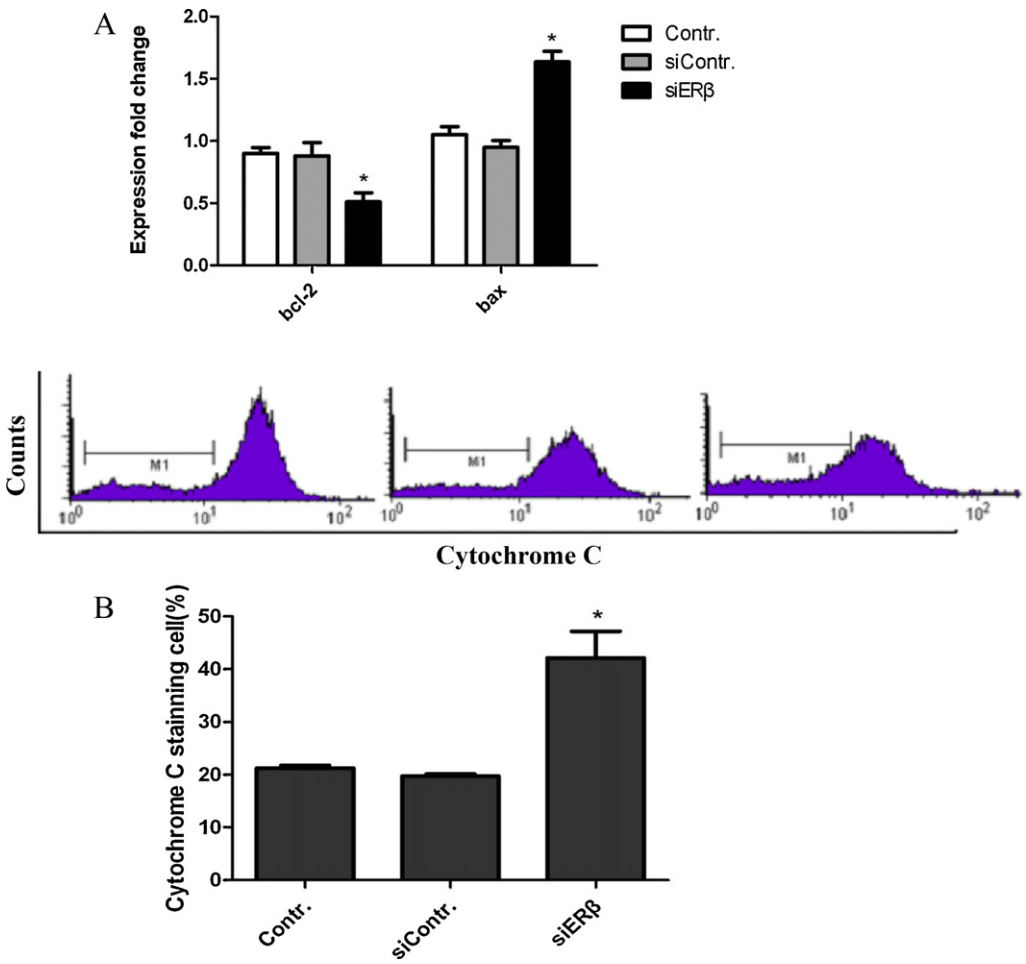

Fig. 6. Transfection of anti-ERβ siRNA can effect bcl2/bax expression and release of cytochrome c in LTEP-a2. (A) Analysis of bcl-2 and bax mRNA expression level by real time-PCR; (B) Cytochrome c releasing analysis by FCM. Graph shows the percentages of Cytochrome c positive cells which represent the mean ± S.E.M from three independent experiments. $^{*}p < 0.05$ vs. control.

with other reports [8,9,22]. A549 cells transfected with ERβ have increased proliferation in response to estrogen [7]. This suggests that there may exist some other pathways to activate ERβ [23,24]. It is reported that SRC-1 is an important cofactor which involves the ligand-independent activity of ERβ [25,26]. So the expression of SRC-1 in SPC-A1 and LTEP-a2 was checked. The western blot results showed that SRC-1 exists in these two cell lines, and its expression increased in pCDNA3.1/ ERβ transfected SPC-A1 (Fig. 3I). But SRC-1 is not detectable in A549 cell (data not shown). Different effects of E2 on lung tumor cells may be attributed to different SRC-1 expression in these cells.

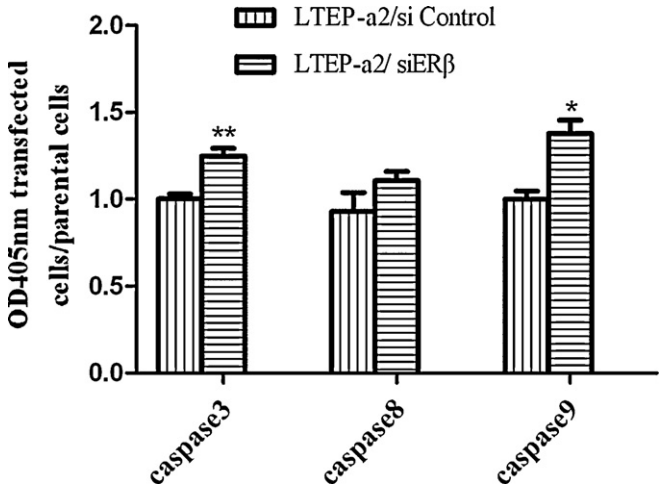

Fig. 7. Colorimetric assay for caspase-3, -8, -9 activities at 72 h after transfection. Data are means ± SE from three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

ERs can bind to estrogen response elements (EREs) in the promoter regions of target genes in a ligand-dependent manner and can also modulate non-ERE-containing genes by interacting with the DNA-bound transcription factors. In addition to transcriptional activation in the nucleus, ERs have non-genomic function by involving rapid activation of many signaling molecules, such as IGF-I and epidermal growth factor (EGF) receptors, MAPK, Akt, protein kinase C [27]. The results of confocal microscopy assay showed that ERβ was localized in nucleus in LTEP-a2 cell. However, except in nucleus, ERβ was also detected in cytoplasm in pCDNA3.1/ ERβ transfected SPC-A1 cells (Fig. 4). It indicates that ERβ mainly exercises its genomic function in LTEP-a2 and both genomic and non-genomic function in ERβ over-expressed SPC-A1 cell.

ERβ involes in apoptosis. The results of Annexin V-PI staining showed that E2 protected LTEP-a2 from apoptosis. Similar results were reported in Retinal Pigmented Epithelium and myocardiac cells [28,29]. After down-regulation of ERβ, the protective contribution of E2 was impaired. It indicates that E2 can arrest NSCLC apoptosis, and its receptor ERβ plays a decisive role in this process.

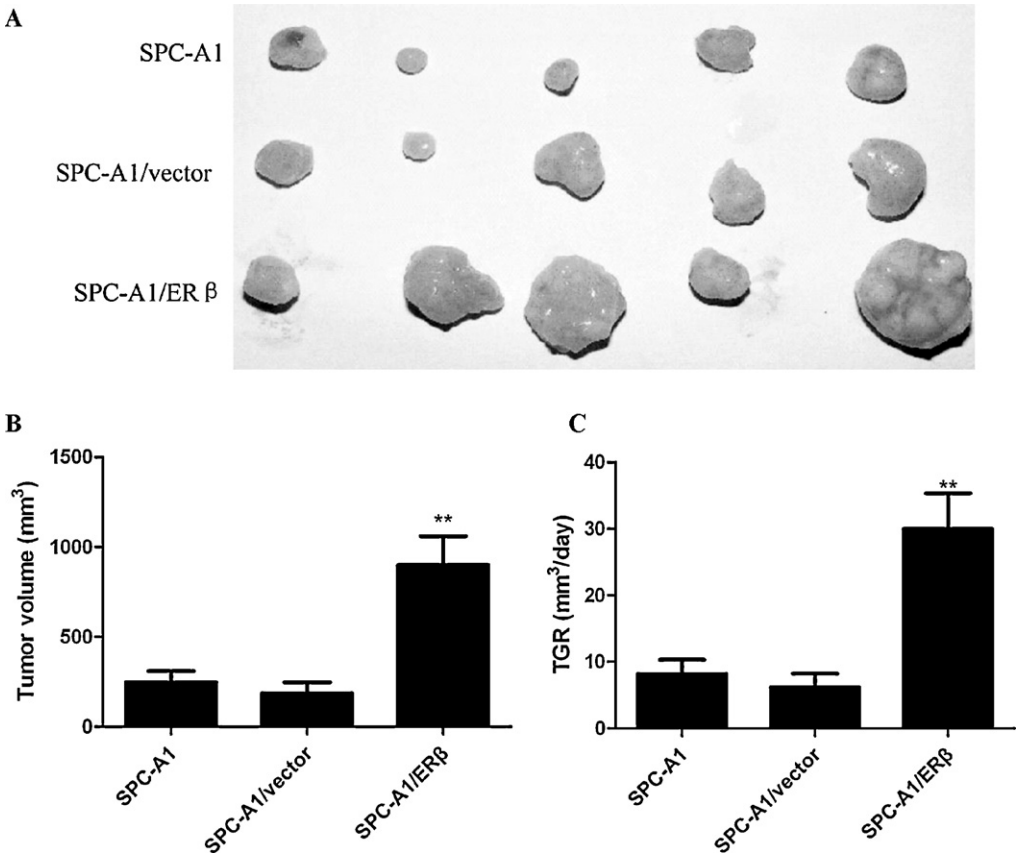

Fig. 8. ERβ transfected cells promote tumorigensis *in vivo*. (A) Photograph of tumor size; (B) measure of tumor volume; (C) analysis of tumor growth rate. $^{**}p < 0.01$ vs. control.

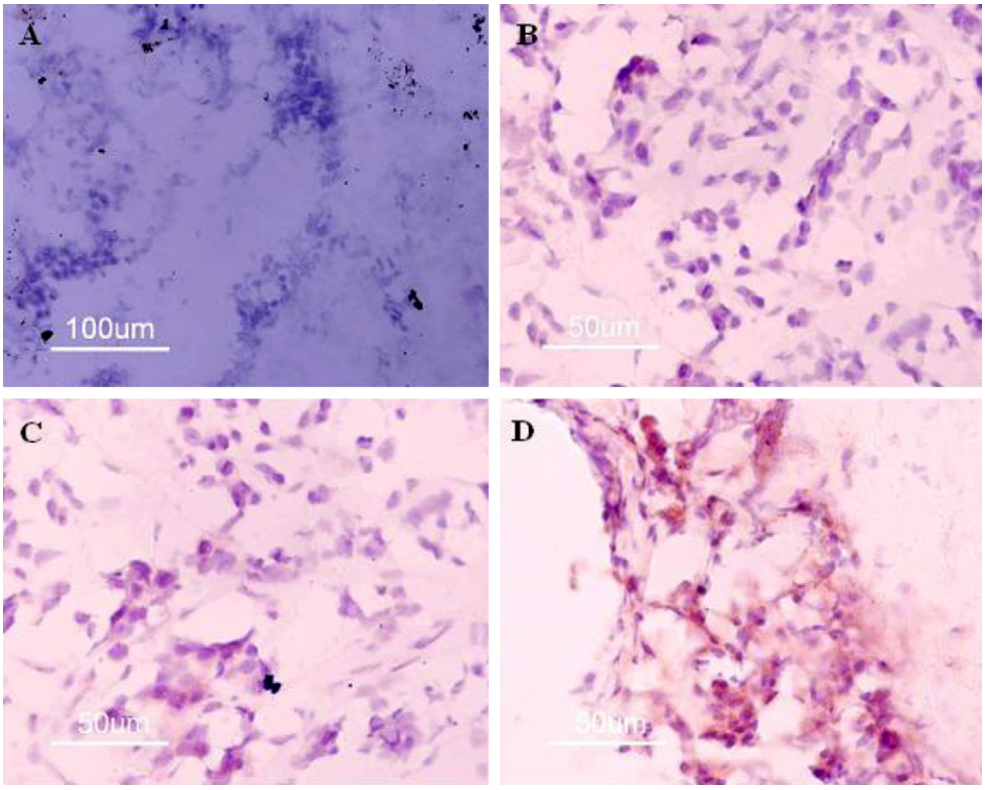

Fig. 9. Immunohistochemical staining of tissues for ERβ with rabbit polyclonal anti-ERβ antibody. (A) Negative control sections; (B) tumor tissue from SPC-A1 injected mice; (C) tumor tissue from SPC-A1/vector injected mice; (D) tumor tissue fromSPC-A1/ ERβ injected mice (×400).

The bcl-2 family proteins have a central role in controlling mitochondria apoptosome-mediated apoptotic pathway. Bcl-2/Bcl-Xl subfamily proteins inhibit apoptosis, while the Bax/Bak subfamily proteins promote cell death [30].After down-regulation of ERβ in LTEP-a2, the expression of bcl-2 was decreased and bax was increased. In addition, ERβ specific siRNA reduced mitochondrial membrane potential and induced the releasing of cytochrome c of LTEP-a2. We also found that the activity of caspase-3 and 9 was increased in LTEP-a2 after transfection of ERβ siRNA. All these data indicated that apoptosis of ERβ siRNA transfected cells might be mediated by the mitochondria-apoptosome-mediated pathway. The down-regulated expression of bcl-2 and up-regulated expression of bax decreased mitochondrial membrane potential, and led to release of cytochrome c, and activated caspase-3 and -9, at last induce the apoptosis of the cells.

ERβ can promote tumorigenesis of NSCLC cells. Colony formation and cell migration experiments are usually used for determining tumor cells' tumorigenesis and migration ability *in vitro* [31,32]. Over-expressed ERβ can significantly enhance the ability of colony formation and cell migration of SPC-A1, while ERβ siRNAs can impaire this ability. In order to further prove ERβ can enhance the tumorigenesis of NSCLC, we inoculated the ERβ-transfected SPC-A1 cells into nude mice. The results demonstrated that ERβ-transfected SPC-A1 cells grew much faster than mock transfected or wild-type cells in nude mice. These results determined that ERβ promoted lung tumorigenesis and also might explain some phenomena from clinical observation. Wu et al. and Skov et al. all showed that ERβ overexpression could be used as a positive prognostic factor for different stage and gender NSCLC patients [33,34]. Kawai et al. concluded that the absence of ER-β could serve as a marker identifying patients at high risk even at an early clinical stage [35].

ERβ can stably express in SPC-A1/ERβ transfected cells in nude mice for a long period. Fig. 9 showed that ERβ was localized in nucleus and cytoplasm of tumor cells. It was consistent with the result of confocal analysis of the ERβ location in SPC-A1/ ERβ transfected cells. However, ERβ was mainly localized in nucleus of LTEP-a2 cells and Chinese NSCLC patients. It indicates that the location of ERβ is cell specific and ERβ can promote tumor proliferation by genomic effects and cytoplasm-non-genomic effects.

In conclusion, ERβ plays an important role in development of Chinese NSCLC and tumorigenesis *in vitro* and *in vivo*. ERβ deactivation or down-regulation may possess potential therapeutic utility for the treatment of lung cancer.

Conflicts of interest statement

None declared.

Acknowledgments

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