

Downregulation of estrogen receptor β inhibits lung adenocarcinoma cell growth

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Abstract. Estrogen receptor β (ER β) is an important ER subtype in lung adenocarcinoma. However, the functions and mechanisms of ER β have not been fully elucidated. The aim of the present study was to investigate the biological effects and relevant mechanisms of ER β in lung adenocarcinoma. The protein expression of ER β was found to be higher in lung adenocarcinoma tissues compared with that in adjacent non-cancerous tissues (n=75, P<0.001). Of note, ER β protein expression was significantly correlated with tumor size (P=0.018), lymph node metastasis (P=0.041), clinical stage (P=0.041) and differentiation (P<0.001). In addition, ER β protein expression in A549 cells was found to be higher compared with that in human bronchial epithelial cells (HBEs). Furthermore, knockdown of ER β expression inhibited colony formation and cell invasion *in vitro*, whereas the number of metastatic tumors in the lungs of mice was decreased *in vivo*. Western blot analysis demonstrated that the expression of phosphorylated extracellular signal-regulated kinase (pERK), matrix metalloproteinase (MMP)-2 and MMP-9 was decreased by downregulation of ER β . Therefore, ER β may play an important role in lung adenocarcinoma progression via the MEK/ERK signaling axis, and it may represent a novel therapeutic target for lung adenocarcinoma in the future.

Introduction

Lung cancer is the most common malignant tumor and one of the main causes of cancer-related mortality worldwide (1).

The 5-year survival rate is ~15%, and only 15-17% of patients diagnosed with distant metastasis survive for 1 year (2,3). Adenocarcinoma is one of the major subtypes of non-small cell lung cancer (NSCLC), and lung adenocarcinoma patients frequently develop metastases (4). It is estimated that >35% of patients with advanced lung adenocarcinoma develop distant metastases, resulting in shorter survival and poor quality of life (5). Conventional treatments, including chemotherapy, radiotherapy and bisphosphonates, have been shown to have limited efficacy (1). Hence, it is crucial to elucidate the mechanisms underlying lung adenocarcinoma progression.

Estrogen and progesterone receptors have been shown to play an important role in NSCLC, particularly lung adenocarcinoma (6). The incidence and mortality of lung cancer were found to be higher among women who receive hormone replacement therapy (7). The effects of estrogen are mediated via estrogen receptors (ER α and ER β) (8). Estrogen receptors are consistently found in lung cancer tissues and cell lines (particularly adenocarcinoma), mostly in the form of ER β (9). Hsu *et al* reported that estrogen promoted lung adenocarcinoma cell proliferation and migration via ER β , and high expression of ER β was identified as an adverse prognostic factor in patients with lung adenocarcinoma (9). However, the detailed mechanism underlying ER β -mediated lung adenocarcinoma progression remains unclear.

The aim of the present study was to determine whether the expression of ER β is higher in lung adenocarcinoma tissues, as well as observe the effects of its knockdown by lentivirus interference RNA on lung adenocarcinoma cell growth and invasion *in vitro* and *in vivo*.

Materials and methods

Immunohistochemical staining of ER β . Tissue microarray (TMA) assays were obtained from Superchip (Shanghai, China) and included 75 cases of tissues from lung adenocarcinoma and adjacent normal tissues (array ID: HLug-Ade150Sur-02). The recorded clinicopathological information included age, sex, tumor size, tumor location and TNM stage. Experiments were performed as described previously (10,11). The tissue sections were de-paraffinized, rehydrated, and treated according to standard protocols (12). Polyclonal rabbit anti-ER β antibody

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(dilution 1:50; cat. no. ab3577) was purchased from Abcam (Cambridge, UK) (13) and incubated at 4°C overnight.

Immunohistochemical assay. The stained tissue arrays were defined as one of nine degrees, according to the immunohistochemical scores reported (10,11). Primarily, six degrees of proportional score for positive staining were assigned according to the proportion of positive tumor cells (0, none; 1, <1/100; 2, 1-10/100; 3, 10-30/100; 4, 30-60/100; and 5, >60/100). Thereafter, four degrees of intensity score were assigned according to the intensity of staining (0, none; 1, weak; 2, intermediate; and 3, strong). The proportion and intensity scores were then added to yield a total score, which ranged from 0 to 8. According to the total score, the cases were classified as low/negative expression (total score, 0-4) and high expression (total score, 5-8). Final scores were confirmed in a double-blind manner by two independent pathologists.

Drugs and chemicals. The ER β agonist diarylpropionitrile (DPN; CAS1428-67-7) and the ER α agonist propylpyrazoletriol (PPT; CAS263717-53-9) were purchased from Tocris Bioscience (Bristol, UK); 17 β -estradiol (E2; MFCD01074033) was purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany).

Cell lines and cell culture. The human lung adenocarcinoma cell line A549, the human breast cancer cell line MCF-7 and human bronchial epithelial (HBE) cells were preserved in our laboratory. The A549, MCF-7 and HBE cells were maintained in RPMI-1640 medium (C11875500B; Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Biochrom GmbH, Berlin, Germany). The cells were incubated at 37°C in a humidified atmosphere of 5% CO₂/95% air.

Western blot analysis. Cells were lysed for protein extraction. After being quantified, 25 μ g of protein was subjected to 10% SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were then blocked in 5% fat-free milk and incubated overnight at 4°C with rabbit polyclonal antibodies against ER β (dilution 1:2,000; cat. no. ab3577), extracellular signal-regulated kinase (ERK)1/2 (dilution 1:500; cat. no. ab176640), pERK1/2 (dilution 1:500; cat. no. ab76299), matrix metalloproteinase (MMP)-2 (dilution 1:2,000; cat. no. ab37150), MMP-9 (dilution 1:2,000; cat. no. ab38898) and mouse monoclonal antibody against GAPDH (dilution 1:8,000; cat. no. ab8245), all purchased from Abcam. Next, the membranes were incubated with anti-rabbit/mouse secondary antibodies (cat. nos. GTX213110-10/GTX213111-01; GeneTex, Inc., Irvine, CA, USA). Finally, the content of the target proteins was determined by chemiluminescence (Pierce; Thermo Fisher Scientific, Inc.).

Cell proliferation assay. The MTT assay (Sigma-Aldrich; Merck KGaA) was conducted to determine cell proliferation. Briefly, A549 cells were seeded into 96-well plates (Corning Inc., Corning, NY, USA) at a density of 1x10⁴ cells/well and treated with E2 (10 nM), DPN (10 nM) or PPT (10 nM) for 0, 24, 48 and 72 h, and added the same volume of phosphate-buffered

saline (PBS) as the control group. At each time-point, 20 μ l MTT (10 mg/ml) was added to each well and successively incubated for another 4 h at 37°C. After removing the supernatant, 150 μ l dimethylsulfoxide (S7020; Invitrogen; Thermo Fisher Scientific, Inc.) was added for 10 min to dissolve the formazan crystals. The absorbance was measured at 490 nm with a microplate reader (Multiskan MK3; Thermo Fisher Scientific, Inc.). Each experiment was performed in triplicate and repeated three times.

Cell infection. Short hairpin RNAs (shRNA1: GCATGGAAC ATCTGCTCAA; shRNA2: GCTGAATGCCCCACGTGCTT; shRNA3: GCAAAGAGGGCTCCCAGAA) targeting ER β (ER β -GV248-RNAi NM_001437, target sequence: GCAAAG AGGGCTCCCAGAA) and control shRNA (NC-GV248, target sequence: TTCTCCGAACGTGTCACGT) were obtained from Shanghai GeneChem Co., Ltd. (Shanghai, China). A549 cells were infected with the ER β -shRNA lentivirus to knock down ER β expression, and NC-shRNA was used at the same time as a negative control group, according to the manufacturer's instructions. After 72 h, green fluorescent protein indicated that the rate of infection was ~90% at a multiplicity of infection of 10. Stably transfected cells were then selected with puromycin for 2 weeks.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) analysis. Total RNA of infected A549 cells was isolated from cells using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. Total RNA (500 ng) was then reverse-transcribed into cDNA (Takara Bio, Inc., Otsu, Japan). RT-PCR was performed using SYBR Premix Ex Taq™ II (Takara Bio, Inc.) and gene expression was quantified using the cycle quantification (Cq) method. The PCR primers were as follows: ER β forward, GAT CATTGCTCCTCTGAGC and reverse, CACCTTCACCGT TCCAGTTT; GAPDH forward, AGCACGGCTCCATATACA TACC and reverse, TGGACCACTAAAGGAGAAAGGT.

Colony formation assay. The infected A549 and control cells in the logarithmic growth phase were harvested and plated into 6-well plates (cat. no. A1098201; BioExcellence International Tech Co., Ltd.) at 500 cells/well. After incubation for 8 days, cell colonies (>50 cells) were stained with 0.25% crystal violet solution and their number was manually counted. Each experiment included three independent biological replicates and each was performed in triplicate.

Cell invasion assay. The invasive potential of the cells was measured using 8- μ m pore size Transwell inserts (Corning, Inc.). The infected A549 and control cells were resuspended in serum-free RPMI-1640 and then seeded in triplicates in the upper chamber covered with 70 μ l Matrigel (diluted in 1:8; Corning, Inc.). Medium (500 μ l) containing 10% FBS was added to the bottom chamber to serve as the chemoattractant. After 24 h, cells that had migrated to the lower chamber were fixed with 95% ethyl alcohol and then stained with 0.5% crystal violet solution. Finally, the number of invading cells was counted in five random fields per sample and the mean was calculated. Each experiment included three independent biological replicates and each was performed in triplicate.

Table I. Correlation of ER β with the clinicopathological characteristics of the lung adenocarcinoma patients.

Characteristics	ER β expression			P-value
	N	Low, n (%)	High, n (%)	
Tissue source				<0.0001
Tumor	75	6 (11.5)	69 (70.4)	
Adjacent	75	26 (88.5)	49 (29.6)	
Age (years)				0.352
<60	32	11 (37.9)	22 (45.7)	
≥ 60	43	19 (62.1)	25 (58.1)	
Sex				0.200
M	40	3 (33.3)	25 (56.1)	
F	35	6 (66.7)	24 (43.9)	
Tumor size (mm)				0.018
≤ 30	31	7 (77.8)	24 (36.4)	
>30	44	2 (22.2)	42 (63.6)	
Location				0.356
Left	31	5 (55.6)	26 (39.4)	
Right	44	4 (44.4)	40 (60.6)	
Differentiation				<0.0001
Well	8	5 (55.6)	3 (4.5)	
Moderate	51	3 (33.3)	48 (72.7)	
Poor	16	1 (11.1)	15 (22.7)	
Lymph node metastasis				0.041
No	27	6 (66.7)	21 (31.8)	
Yes	48	3 (33.3)	45 (68.2)	
Stage				0.041
I-II	43	8 (88.9)	35 (53.0)	
III	32	1 (11.1)	31 (47.0)	

ER, estrogen receptor; M, male; F, female.

In vivo experiments. An experimental model of A549 cell lung metastasis was constructed to study the effects of ER β on lung adenocarcinoma *in vivo*. A total of 18 female NOD-SCID mice, aged 4 weeks and weighing 20-25 g, were purchased from Beijing HFK Bioscience Co. (Beijing, China). They were housed in a specific pathogen-free (SPF) laboratory animal environment (temperature, 22°C; ventilation rate, 15/h; light/dark cycle, 12/12 h; food was sterilized with Cobalt-60 irradiation and water was autoclaved, and access to the food was *ad libitum*; tumor size not exceed 2.0 cm) by professional breeders and randomly divided into three groups (6 mice/group). A549, A549-ER β -shRNA and A549-NC-shRNA cells ($1 \times 10^6/200 \mu\text{l}$) were harvested, resuspended in PBS and injected via the tail vein. After 3 days, E2 (0.1 mg/kg) was subcutaneously injected once a week in a volume of 100 μl per mouse. When the experimental mice developed symptoms such as lameness, joint stiffness, decreased exercise capacity, paraplegia, or an experiment for 42 days, the experiment required termination. The mice were sacrificed humanely in a transparent euthanasia device (ventilated 10% of isoflurane for 1 min before laying the

mice, and constantly ventilated isoflurane for another 3 min after the mice were dead). The lungs were then excised and weighed; the number of the metastatic lesions larger than 0.5 mm in diameter on the surface of the lungs was counted, fixed in 10% formalin, embedded in paraffin, and sectioned for H&E staining.

All animal studies strictly abided by the Regulations on Animal Experimentation formulated by the Laboratory Animal Center of the Fourth Military Medical University (The Air Force Medical University) (Xi'an, China) and the present study was approved by the Animal Experimental Ethical Inspection Committee of this Center (no. 20170803).

Statistical analysis. Statistical analyses were conducted using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Wilcoxon rank sum test was used for immunohistochemical total scores analysis and the p-ERK/ERK ratio analysis was conducted by t-test. The other data were analyzed by one-way analysis of variance (ANOVA) with least significant difference (LSD) test as the post hoc test. P-values <0.05 were considered to indicate statistically significant differences.

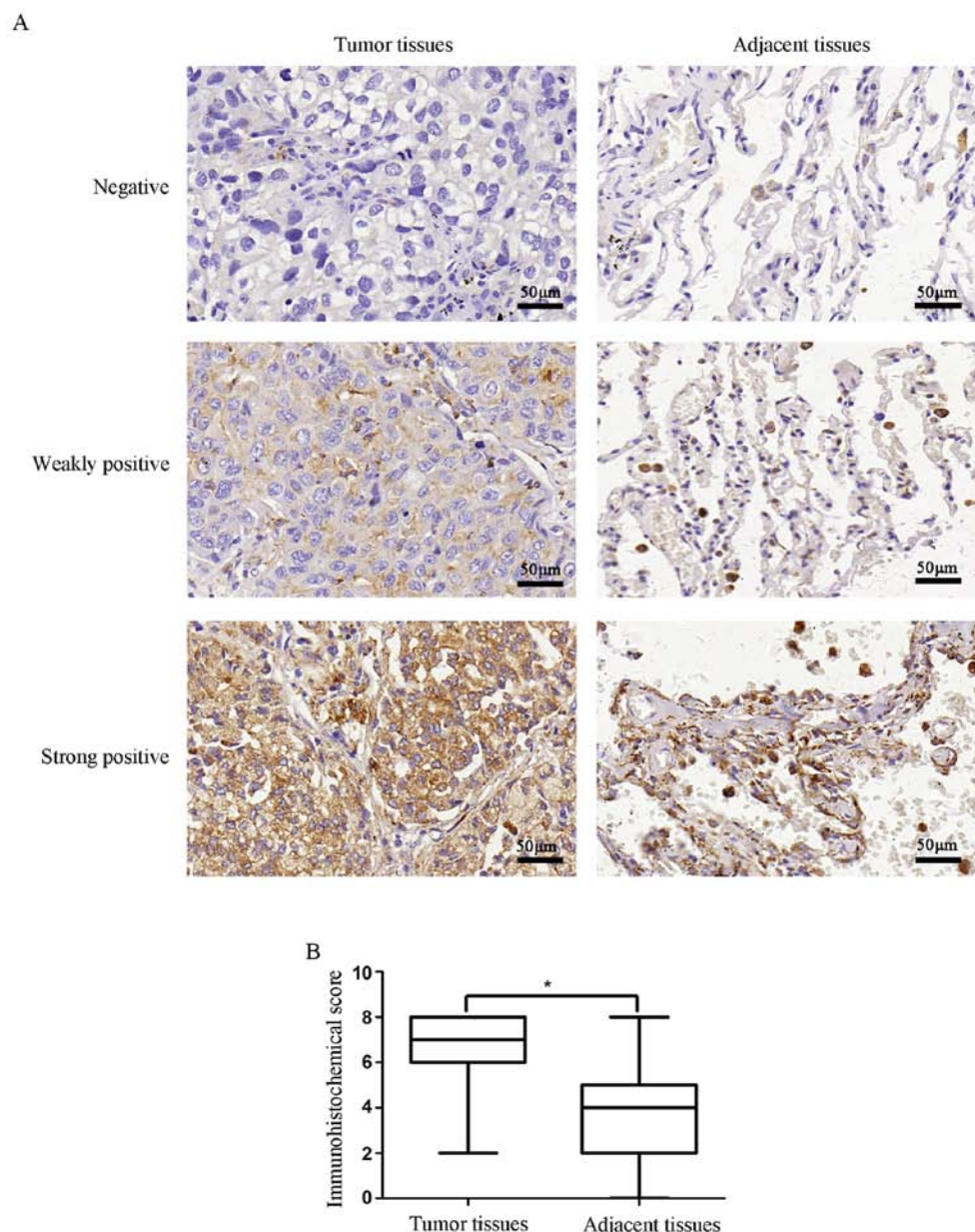


Figure 1. Estrogen receptor (ER) β is overexpressed in lung adenocarcinoma tissues. (A) Representative immunohistochemical staining patterns of ER β (magnification, x400; scale bar, 50 μ m). (B) Total immunohistochemical score of the relative expression of ER β in lung adenocarcinoma and adjacent non-cancerous tissues (n=75). *P<0.05 compared with the adjacent non-cancerous tissues.

Results

Expression of ER β in lung adenocarcinoma patient samples and cell lines. Immunohistochemistry was used to evaluate the association of ER β expression and pathological characteristics with a TMA that consisted of 75 paired lung adenocarcinoma specimens and corresponding normal samples. The expression of ER β was observed in the cytoplasm. The correlations of ER β expression with clinicopathological characteristics in the lung adenocarcinoma patients are presented in Table I and Fig. 1. We observed that the protein expression of ER β was higher in lung adenocarcinoma tissues compared with that in adjacent non-cancerous tissues (P<0.001). Notably, ER β protein expression was significantly correlated with tumor size (P=0.018), lymph node metastasis (P=0.041), clinical stage (P=0.041) and tumor differentiation (P<0.001). The results indicated that the

expression of ER β may be associated with the occurrence and progression of lung adenocarcinoma.

Furthermore, ER β expression was compared among lung adenocarcinoma cells (A549), breast cancer cells (MCF-7) and HBE cells. MCF-7 cells were used as the positive control, and ER β expression was found to be higher in A549 compared with that in MCF-7 and HBE cells (Fig. 2). The results indicated that ER β is an important functional ER subtype in A549 cells.

E2 and DPN promote A549 cell proliferation. The effects of E2, DPN and PPT on A549 and MCF-7 cells were investigated. MTT assay was conducted to evaluate cell proliferation. As shown in Fig. 3A, the proliferation of cells treated with E2 and DPN was markedly increased compared with that in the control groups while PPT which was effective for the

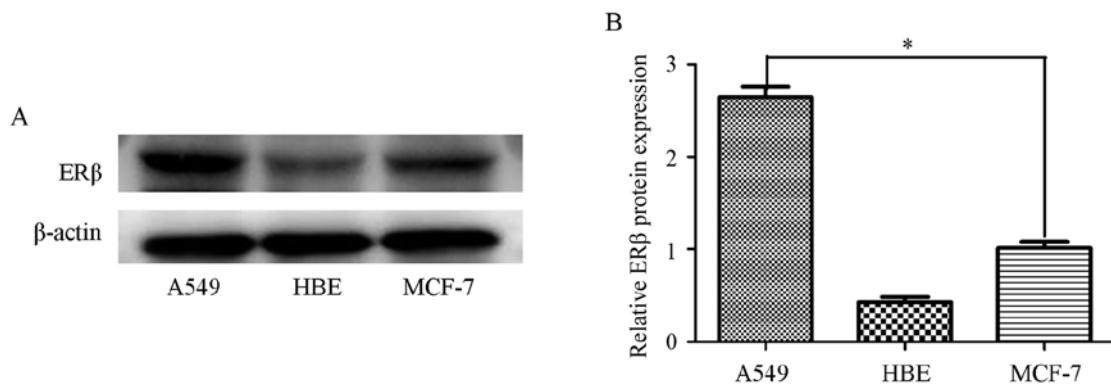


Figure 2. (A and B) Expression of estrogen receptor (ER)β in A549, MCF-7 and human bronchial epithelial (HBE) cells. MCF-7 cells were used as the positive control. Data are presented as the mean ± standard deviation of three replicates. *P<0.05 compared with HBE cells.

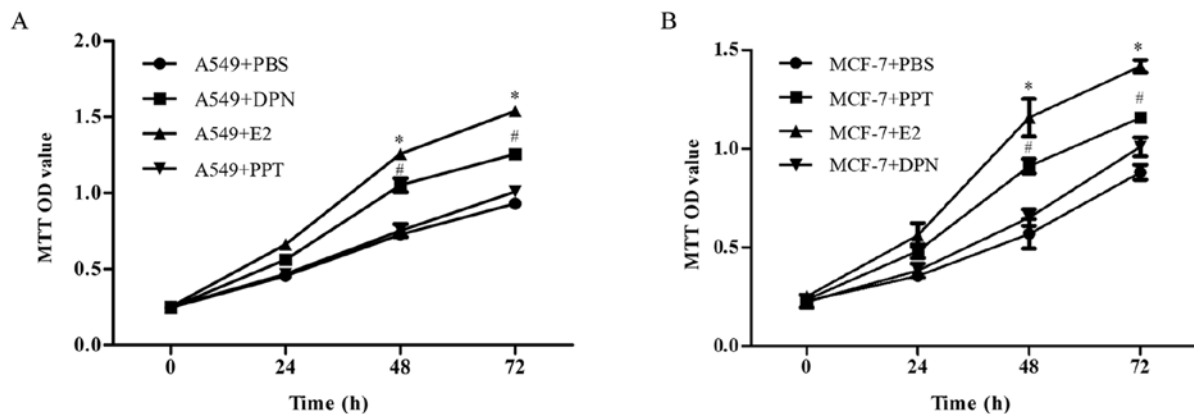


Figure 3. E2 and DPN promote A549 cell proliferation. Cells were treated with E2, DPN, PPT or PBS for 0, 24, 48 and 72 h, and cell proliferation was evaluated using the MTT assay. (A) Effect of E2, DPN and PPT on the A549 cell proliferation. (B) Effect of E2, DPN and PPT on the MCF-7 cell proliferation. Data are presented as the mean ± standard deviation. *P<0.05 (E2 group vs. control group) and #P<0.05 (DPN group vs. control group). DPN, diarylpropionitrile; PPT, propylpyrazoletriol; E2, 17β-estradiol; PBS, phosphate-buffered saline.

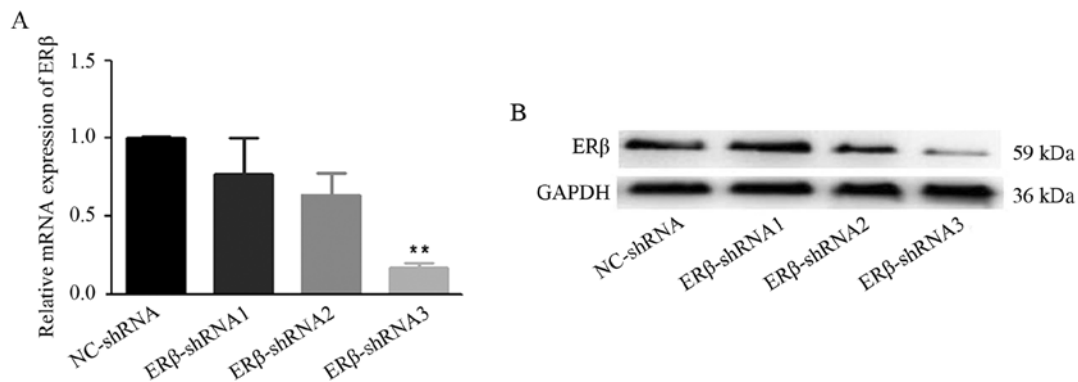


Figure 4. Effect of lentivirus-mediated knockdown of estrogen receptor (ER)β expression in A549 cells. (A) The ERβ mRNA levels in A549 cells infected with ERβ-shRNA or NC-shRNA were evaluated with reverse transcription-polymerase chain reaction analysis (*P=0.0145, ERβ-shRNA3 compared with the NC-shRNA group). (B) Western blot analysis of ERβ protein expression in A549 cells infected with ERβ-shRNA and NC-shRNA.

(AMCF-7 cells (Fig. 3B) did not obviously promote A549 cell proliferation; the E2-induced A549 cell proliferation may be mediated via ERβ more than ERα.

Expression of ERβ decreases in cells stably transfected by lentivirus RNA interference. The lentivirus RNA interference technique was used to downregulate the expression of ERβ.

Following cell infection and antibiotic screening for 2 weeks, the infection efficacy was confirmed by RT-PCR (Fig. 4A) and western blot analysis (Fig. 4B and C). ERβ was stably decreased by shRNA and the ERβ-GV248-RNAi#3 was the most effective one, thus we named it as ERβ-shRNA for the following tests. The results indicated that ERβ expression was suppressed by ERβ-shRNA.

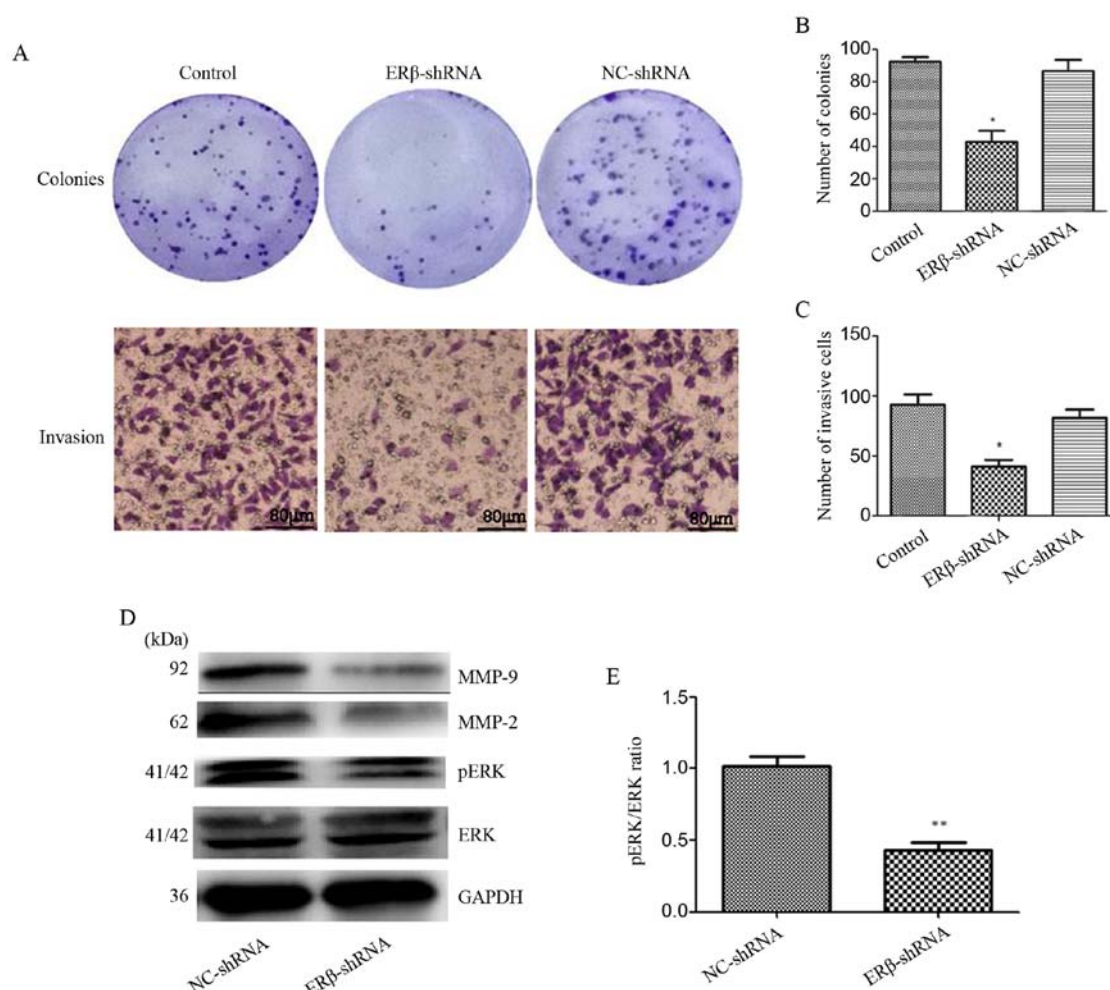


Figure 5. Downregulation of estrogen receptor (ER β) expression inhibits colony formation and invasion of A549 cells *in vitro*. (A) Representative images of cell colony formation and invasion. (B) The number of colonies was calculated. (C) The number of invading cells was calculated (magnification, x100). (D) Western blot analysis of the expression of extracellular signal-regulated kinase (ERK), pERK, matrix metalloproteinase (MMP)-2 and MMP-9. GAPDH was used as the endogenous control. (E) pERK/ERK ratio was analyzed and data are presented as the mean \pm standard deviation of three replicates. * $P < 0.05$ or ** $P < 0.01$, compared with the NC-shRNA group. NC, negative control.

Downregulation of ER β expression inhibits colony formation and invasion of A549 cells *in vitro*. The effects of ER β -shRNA on A549 cells were examined. As shown in Fig. 5A-D, colony formation and cell invasion assays demonstrated that ER β -shRNA inhibited A549 cell proliferation and invasion compared with NC-shRNA and control ($P < 0.05$). Mechanistically, ER β knockdown suppressed the expression of pERK, MMP-2 and MMP-9 (Fig. 5E; $P < 0.05$). These findings indicate that ER β is a functional mediator. Therefore, knockdown of ER β expression inhibited A549 cell proliferation and invasion via downregulation of pERK, MMP-2 and MMP-9.

Downregulation of ER β expression suppresses lung metastasis of A549 cells *in vivo*. To investigate whether downregulation of ER β expression may serve as a therapeutic target for lung adenocarcinoma, an experimental lung metastatic mouse model was constructed. *In vivo* lung metastasis assay demonstrated that downregulation of ER β expression was associated with fewer metastatic tumors and lower lung weight compared with the control (Fig. 6). These results indicated that downregulation of ER β may inhibit tumor growth and lung metastasis *in vivo*.

Discussion

Estrogen and ERs are considered to play an important role in lung carcinogenesis (14). Several studies have demonstrated that ER β is the predominant ER in lung cancer tissue and tumor cell lines, particularly adenocarcinoma (15,16). It was previously reported that estrogen promotes lung adenocarcinoma cell proliferation and migration via ER β (11). However, the detailed mechanism underlying ER β -mediated lung adenocarcinoma progression remains unclear. The present study was designed to investigate the biological effects and mechanism of action of ER β in lung adenocarcinoma.

To determine the association between ER β and clinicopathological characteristics in lung adenocarcinoma, immunohistochemistry was used to evaluate the expression of ER β in TMA, which included 75 tumor and adjacent normal tissues from patients with lung adenocarcinoma. A higher ER β expression was detected in lung adenocarcinoma specimens compared with adjacent non-cancerous tissues. Notably, ER β protein expression was found to be significantly correlated with tumor size ($P = 0.018$), lymph node metastasis ($P = 0.041$), clinical stage ($P = 0.041$) and tumor differentiation ($P < 0.001$),

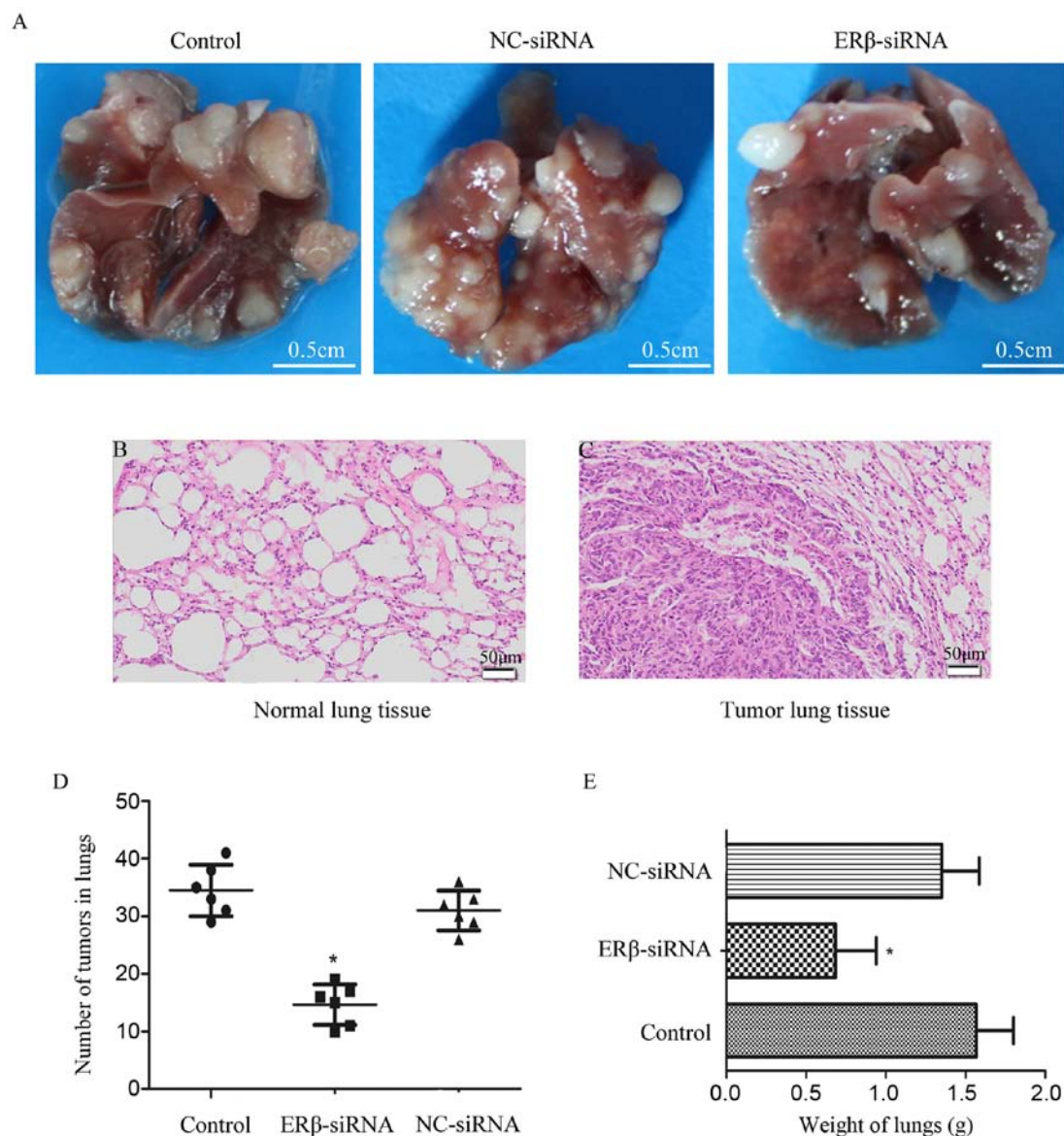


Figure 6. Downregulation of estrogen receptor (ER) β expression suppresses lung metastasis of A549 cells *in vivo*. (A) Representative images of the lungs removed after treatment (n=6/group). (B) Representative pathological image of normal lung tissue (magnification, x200). (C) Representative pathological image of metastatic tumors in the lungs (magnification, x100). (D) Quantification of metastatic tumors in the lungs of each group at 4 weeks after treatment. (E) Quantification of lung weight at 4 weeks after treatment. Data are presented as the mean \pm standard deviation of three replicates. *P<0.05, compared with control and NC-shRNA groups. NC, negative control.

suggesting that ER β plays an important role in the occurrence and development of lung adenocarcinoma. Our findings were similar to those of Luo *et al*, who reported that ER β overexpression promotes the progression of NSCLC (16).

Our findings *in vitro* were in agreement with the TMA results. ER β expression was higher in the A549 cell line compared with that in HBE cells. ER β was identified as the important ER subtype in lung adenocarcinoma cells. MTT assay was used to investigate whether the effects of E2 are mediated via ER α or ER β . The results indicated that E2 induced lung adenocarcinoma cell proliferation via ER β . These results are supported by the findings of Fan *et al* (17) and Warner and Gustafsson (18), who reported that ER α was not the main mediator of transcriptional responses to E2 in NSCLC cells, but ER β was more likely to be the primary type in lung tumor tissues and cell lines, and that estrogen-dependent responses in

NSCLC cells are principally mediated by ER β . Furthermore, we demonstrated that downregulation of ER β expression inhibited colony formation and invasion of A549 cells. *In vivo*, we constructed an experimental lung metastatic mouse model. The lung metastasis assay demonstrated that downregulation of ER β expression was associated with fewer metastatic tumors and lower lung weight compared with the control group. Our results are consistent with those of Fan *et al*, who reported that estrogen and DPN promote lung metastasis of A549 cells (17). Fan *et al*'s study revealed that estrogen induced lung cancer metastasis through the ER β /MMP-2 axis. However, in the present study, MMP-9 was identified as a novel target gene of ER β . Thus, ER β may promote lung cancer metastasis not only through MMP-2, but also through MMP-9. The development of lung adenocarcinoma is a complex multistep process. The ERK signaling pathway plays an important role in lung cancer cell proliferation

and invasion. Therefore, the protein expression of ERK and pERK was determined in A549-ER β -shRNA cells. The results revealed that downregulation of ER β expression decreased pERK expression levels. This finding suggested that downregulation of ER β expression inhibited the proliferation and invasion of A549 cells through suppressing the phosphorylation of ERK. ERs also regulate gene expression through binding to other transcription factors, such as the activator protein 1 (AP-1) (19), the most important structural components of which are c-Jun and c-Fos. In addition, MMP is crucial for malignant tumor metastasis (20,21), particularly MMP-2 and MMP-9.

Taken together, the findings of the present study indicate that ER β may promote lung adenocarcinoma growth and metastasis through the MEK/ERK signaling pathway and MMP-2/MMP-9 expression, and therefore, hold promise as a novel therapeutic target for lung adenocarcinoma.

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Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

HZ designed the study. WC and BX performed the immunohistochemical assay and all the *in vitro* experiments and collected the data. HP, LH and WS conducted the animal experiments and collected the data. PC, LD, ZZ and LL analyzed the data and performed the relative statistical analysis. ZZ and LL provided guidance during the study. WC contributed to the writing of the manuscript. All authors have read and approved the final version of this manuscript and agree to be accountable for all aspects of the research in ensuring that the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Ethics approval and consent to participate

All animal studies strictly abide by the Regulations on Animal Experimentation formulated by the Laboratory Animal Center of the Fourth Military Medical University (The Air Force Medical University) (Xi'an, China) and this study was approved by the Animal Experimental Ethical Inspection Committee of this Center (no. 20170803).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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