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Effects of Estrogen-related Receptor alpha (ERRα) on Proliferation and Metastasis of Human Lung Cancer A549 Cells*

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Summary: Estrogen-related receptor alpha (ERRα) plays an important role in the development of hormone-dependent cancers, but its roles in lung cancer remain elusive. The present study was aimed to investigate the effects of ERRα on the proliferation and metastasis of lung cancer A549 cells. The mRNA and protein levels of ERRα were detected in lung cancer A549 and MCF-7 cells and bronchial epithelial BEAS-2B cells by qRT-PCR and Western blotting, respectively. ERRα plasmid transfection and XCT-790 (an inverse agonist of ERRα) were used to up-regulate or down-regulate ERRα expression in A549 cells, respectively. The viability of A549 cells was measured by cell counting kit-8 (CCK-8) and the motility of A549 cells by wound healing assay and Transwell migration/invasion assay. The epithelial markers E-cadherin (E-Cad) and zona occludin-1 (ZO-1), the mesenchymal markers fibronectin (FN) and vimentin (Vim) and the transcription factors (Snail, Zeb1 Twist and Slug) were further detected at mRNA and protein levels by qRT-PCR and Western blotting, respectively. The results showed that ERRα promoted the growth of lung cancer A549 cells *in vitro*. XCT-790 significantly inhibited the migration and invasion of A549 cells. Over-expression of ERRα promoted the epithelial-to-mesenchymal transition (EMT) of A549 cells, down-regulated the epithelial makers E-Cad and ZO-1, and up-regulated the mesenchymal makers FN and Vim. Silencing of Slug, but not other transcription factors, significantly abolished the ERRα-induced EMT of A549 cells. It was suggested that ERRα promoted the migration and invasion of A549 cells by inducing EMT, and Slug was involved in the process. Targeting ERRα might be an efficient approach for lung cancer treatment.
Key words: estrogen-related receptor alpha; XCT-790; migration; invasion; A549 cells

Estrogen-related receptor alpha (ERRα) is an orphan nuclear receptor that does not bind to natural estrogens or any other endogenous ligand[1]. It has been identified in a variety of organs including skeletal muscle, kidney, heart, liver, and adipose[2]. Previous studies indicated that ERRs and estrogen receptors (ERs) share common target genes such as pS2, lactoferrin, aromatase, and osteopontin and cross-talk with each other[3, 4]. ERRα can bind to ERR-response elements (ERREs) and then activate their transcription. The ERREs are different from those mediating the estrogenic response (estrogen response elements, EREs)[5]. Recent studies indicated that ERRα may interfere with the estrogen/androgen-related signaling pathways and then play an important role in the development of hormone-dependent cancers[6].

ERRα is found to express in multiple human cancer cells such as breast[7], colorectal[8], and ovarian[9] cancer cells. Its expression is positively correlated with the risk of recurrence and poor clinical outcomes of breast[10] and ovarian cancer[11]. Further, recent studies revealed that targeting ERRα can inhibit the growth of breast[12] and colorectal[13] cancer and suppress the epithelial-to-mesenchymal transition (EMT) and stem cell properties of ovarian cancer cells[9]. It is suggested that ERRα can directly regulate tumor progression of various cancer cells.

Lung cancer causes greatest cancer-related deaths in both men and women worldwide[14]. Previous studies revealed that ERs are expressed in lung cancer cells and the estrogen can promote the progression of lung cancer both *in vitro* and *in vivo*[15, 16], which suggested that estrogenic signal pathway plays an important role in tumorigenesis and progression of lung cancer. However, the roles of ERRα in the development of lung cancer have not yet been elucidated.

In the present study, we demonstrated that ERRα was highly expressed in lung cancer A549 cells and it promoted the proliferation and migration of A549 cells. Furthermore, the over-expression of ERRα induced the EMT of A549 cells by up-regulating the transcription factor Slug.

1 MATERIALS AND METHODS

1.1 Reagents and Materials

All chemicals were of reagent grade or better and were purchased from Sigma Chemical Co. (USA) unless

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other specifically stated. XCT-790, the inverse agonist of ERRα, was dissolved in dimethyl sulfoxide (DMSO) to prepare a 10 mmol/L stock solution, and stored at –20°C. Primary antibodies against E-cadherin (E-cad), zona occludin-1(ZO-1), N-cadherin (N-Cad), fibronectin (FN), vimentin (Vim), Snail, Slug, Zeb1, Twist, and GAPDH were purchased from Cell Signaling Technology (USA). Horseradish peroxidase-conjugated secondary antibody was from Santa Cruz Biotechnology (Santa Cruz, USA). All compounds were solubilized in DMSO. PrimeScript® RT reagent kit and SYBR® Premix Ex Taq™ were products of TaKaRa (Takara Shuzo Co. Ltd., Japan). E.Z.N.A® HP Total RNA kit was bought from Omega Bio-Tek (USA). Medium containing 0.5% DMSO was used as the control.

1.2 Cell Culture and Transfection

Human cell line A549, MCF-7, and bronchial epithelial BEAS-2B cells were obtained from the American Type Culture Collection. The cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 g/mL streptomycin at 37°C in a humidified 5% CO_2 atmosphere. Twenty-four h before the experiments, the medium was removed and replaced with RPMI 1640 without phenol red but supplemented with 5% dextran-coated, charcoal-treated FBS (5% DC-FBS) to exclude estrogenic effects caused by the medium. Flag-tagged ERRα and its control vector were purchased from Addgene (USA). To over-express ERRα, cells were transiently transfected with 1 μg plasmid DNA per well in six-well plates by using lipofectamine 2000 reagent (Invitrogen, USA), and incubated for 24 h according to the manufacturer's instructions. For RNA interference, A549 cells were transfected with 100 pmol siRNA oligomer mixed with lipofectamine 2000 reagent in serum-reduced medium according to the manufacturer's instructions. The target sequences for Snail siRNAs (si-Snail) were 5'-UGCAGUUGAAGAUCUUCCGCGACUG-3'; si-Slug 5'-GAG GAA AGA CTA CAG TCC AAG-3'; si-ERRα 5'-ATC GAG AGA TAG TGG TCA CCA TCA G-3'; negative control siRNA (si-NC) 5'-CAG CUU UGG CUG AGC GUA U-3'. All the siRNA products were obtained from Ambion (USA).

1.3 Cell Viability Assay

The A549 cells (1×10^4) were seeded into each well of a 96-well plate, and subsequently treated with XCT-790 or transfected with ERRα plasmid. Then, the cell viability was assessed by use of cell counting kit-8 (CCK-8) according to the manufacturer's instructions (Dojindo Molecular Technologies, Japan). Briefly, CCK-8 solution (10 μL) was added and incubated at 37°C for 1 h, and the absorbance (*A*) value of each well was read at 450 nm using a microplate reader (Spectra MAX 340, Molecular Devices Co., USA).

1.4 Wound Healing Assay

For the *in-vitro* wound healing assay, confluent monolayers of A549 cells treated with XCT-790 were scratched, and the migration distance of the cells into the scratched area was measured in 5 randomly chosen fields. Following the indicated time of culture in RPMI-1640 supplemented with 2% serum (control) or XCT-790, the migration of the cells was evaluated by measuring the difference in the area of the wounds with a Leica DM2500 image analysis system (Leica, Germany).

1.5 Transwell Migration/Invasion Assay

Migration and invasion assays were performed in Boyden chambers. The polycarbonate filters (8 μm pore size, Corning, USA) pre-coated with Matrigel Matrix (BD Biosciences, USA) were used for invasion assay, and uncoated filters were used for migration assay. A549 cells (1×10^5) that had been treated with the control medium or with medium containing XCT-790 were added to transwell chambers. Serum was added to the bottom wells of the chambers to induce cell migration and invasion. After incubation for the indicated time, cells that had migrated and invaded through the membrane were stained with 0.5% methylrosaniline chloride solution and counted under an upright microscope (5 fields per chamber). Each migration and invasion assay was repeated in three independent experiments.

1.6 Western Blot Analysis

Cells were lysed in cell lysis buffer [containing 1% NP-40, 20 mmol/L Tris-HCl (pH 7.6), 0.15 mol/L NaCl, 3 mmol/L EDTA, 3 mmol/L EGTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 20 mg/mL aprotinin, and 5 mg/mL leupeptin], and then lysates were cleared by centrifugation and denatured by boiling in Laemmli buffer. Equal amounts of protein (20 μg) were run on 10% SDS polyacrylamide gels, and transferred to nitrocellulose membranes. Following blocking with 5% non-fat milk at room temperature for 2 h, membranes were incubated with the primary antibody at 1:1000 dilution overnight at 4°C and then incubated with a horseradish peroxidase-conjugated secondary antibody at 1:5000 dilution for 2 h at room temperature. Specific immune complexes were detected using Western Blotting Plus Chemiluminescence Reagent (Life Science, Inc., USA).

1.7 Quantitative Real-Time PCR

A549 cells (1×10^6) were plated on 6-well plates. After transfected with ERRα plasmid for 24 h, cells were washed twice with ice-cold PBS. Total mRNA was extracted with TRIZOL reagent. First strand of cDNA was generated from 2 μg total RNA using oligo-dT primer and Superscript Ⅱ Reverse Transcriptase (GIBCO BRL, USA). Quantitative Real-Time PCR was run on an iCycler (Bio-rad, USA) using validated primers and SYBR Premix Ex Taq Ⅱ (Takara, Japan) for detection. The cycle number at which the fluorescence first reached a preset threshold (Ct) was used to quantify the initial concentration of individual templates for expression of mRNA of genes of interest. Transcripts of the housekeeping gene GAPDH in the same incubations were used for internal normalization. Primer pairs were as follows: GAPDH, forward 5'-GCA CCG TCA AGG CTG AGA AC-3' and reverse 5'-TGG TGA AGA CGC CAG TGG A-3'; ERRα, forward 5'- TCC AGC TCC CAC TCG CTG CC -3' and reverse 5'- ACA CTC GTT GGA GGC CGG AC -3'; Snail, forward 5'-GAC CAC TAT GCC GCG CTC TT-3' and reverse 5'-TCG CTG TAG TTA GGC TTC CGA TT-3'; Zeb1, forward 5'-TAC AGA ACC CAA CTT GAA CGT CAC A-3' and reverse 5'-GAT TAC ACC CAG ACT GCG TCA CA-3'; Twist, forward 5'-GGA GTC CGC AGT CTT ACG AG-3' and reverse 5'-TCT GGA GGA CCT GGT AGA GG-3'; Slug, forward 5'-TTC GGA CCC ACA CAT TAC CT-3' and reverse 5'-GCA GTG AGG GCA AGA AAA AG-3'. Transcripts of the housekeeping gene GAPDH in the same

incubations were used for internal normalization.

1.8 Statistical Analysis

All values were reported as $\bar{x}\pm s$ of three independent experiments unless otherwise specified. Data were analyzed by two-tailed unpaired Student's *t*-test between two groups. The statistical analyses were performed using SPSS 17.0 for Windows. A $P<0.05$ was considered to be statistically significant.

2 RESULTS

2.1 Effects of ERRα on Proliferation of A549 Cells

To understand the role of ERRα in lung cancer, its expression was detected in A549 cells and compared with the positive control (MCF-7 cells) and human bronchial epithelial BEAS-2B cells. The results showed that both protein (fig. 1A) and mRNA (fig. 1B) levels of ERRα in A549 cells were comparable with those in MCF-7 cells, greater than those in BEAS-2B cells, suggesting that ERRα is highly expressed in lung cancer A549 cells. Furthermore, XCT-790, the inverse agonist of ERRα, inhibited the proliferation of A549 cells in a dose-dependent manner at both 24 and 48 h (fig. 1C). The IC_{50} values of XCT-790 to A549 cells were 5.5 μmol/L and 2.6 μmol/L at 24 and 48 h, respectively. The results of ERRα knockdown experiments by use of si-ERRα revealed that the viability of cells transfected with si-ERRα was significantly decreased when compared with that of cells transfected with si-NC (negative control) (fig. 1D). Additionally, our results showed that over-expression of ERRα by transfection of ERRα plasmid significantly promoted the proliferation of A549 cells in a time-dependent manner (fig. 1E). Collectively, our results revealed that ERRα can promote the growth of lung cancer A549 cells *in vitro*.

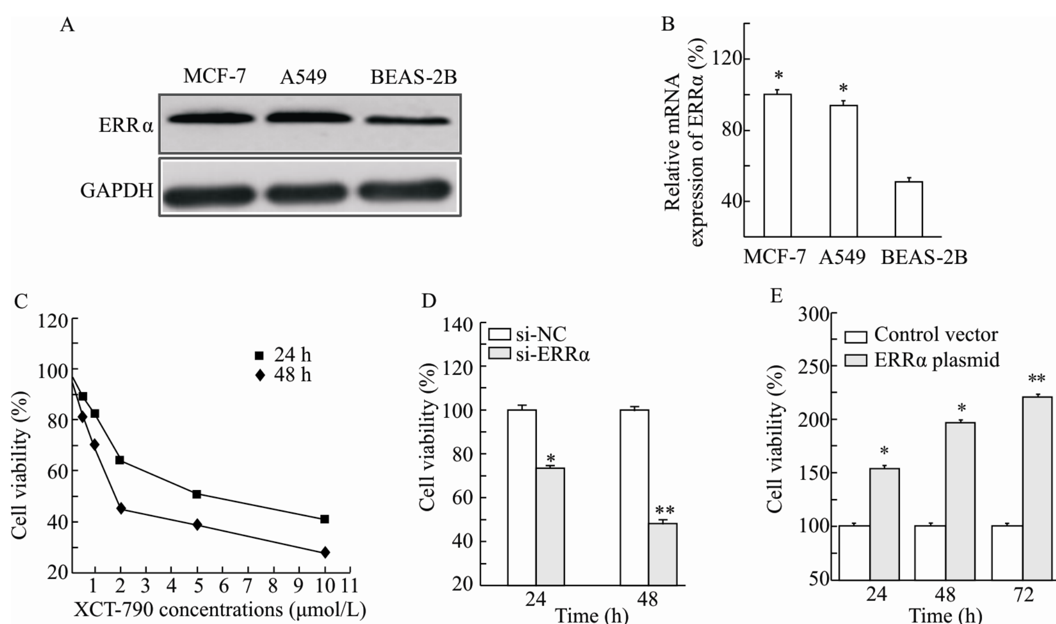

Fig. 1 Effects of ERRα expression on proliferation of A549 cells

A: Western blot analysis of the expression of ERRα in A549, MCF-7 and BEAS-2B cells; B: RT-PCR analysis of the relative expression of ERRα in A549, MCF-7 and BEAS-2B cells, $^{*}P<0.05$ *vs*. BEAS-2B cells; C: cell viability of A549 cells treated with increasing concentrations of XCT-790 for 24 and 48 h, respectively; D: cell viability of A549 cells transfected with si-RNA negative control (si-NC) or si-ERRα at 24 and 48 h, respectively, $^{*}P<0.05$, $^{**}P<0.01$ *vs*. si-NC; E: cell viability of A549 cells transfected with the ERRα plasmid or the control vector at 24, 48 and 72 h, respectively, $^{*}P<0.05$, $^{**}P<0.01$ *vs*. control vector. Data are presented as $\bar{x}\pm s$ of three independent experiments.

2.2 Roles of ERRα in Motility of A549 Cells

The effects of ERRα on the motility of A549 cells were further investigated by use of wound healing and Transwell migration/invasion assays. As shown in fig. 2A, treatment with 1 μmol/L XCT-790 for either 48 or 72 h significantly decreased wound closure of A549 cells as compared with the control group with the difference being statistically significance between the two groups ($P<0.05$) (fig. 2B).

Furthermore, exposure to 1 μmol/L XCT-790 resulted in a significant decrease in the migrating (fig. 2C) and invading abilities of cells (fig. 2D). The number of migrating and invading cells were significantly less in XCT-790 group than in control group at 48 h or 72 h ($P<0.05$). These results suggested that the inverse agonist of ERRα can significantly inhibit the migration and invasion of A549 cells.

2.3 Roles of ERRα in EMT of A549 Cells

EMT has been considered to be the first step of tumor invasion and metastasis[17], which occurs at the invasive front of lung cancer with the down-regulation of epithelial makers E-cad and ZO-1 and the up-regulation of mesenchymal markers N-cad and FN[18]. As shown in fig. 3A, over-expression of ERRα for 48 h promoted the cell-to-cell contacts and induced a fibroblast-like change.

Subsequently, the protein levels of EMT biomolecular markers were measured by Western blot analysis. The results (fig. 3B) showed that transfection with ERRα plasmid at 48 h significantly down-regulated the epithelial makers E-Cad and ZO-1 and up-regulated

the mesenchymal makers Vim, N-Cad and FN, suggesting that the over-expression of ERRα can promote the EMT of lung cancer A549 cells.

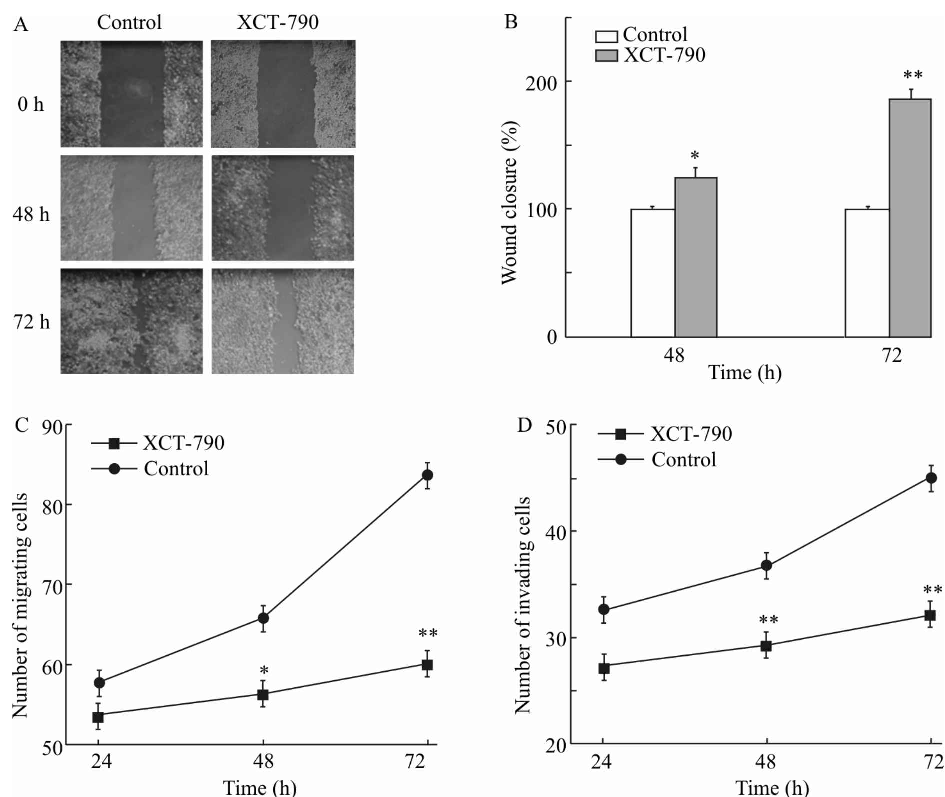

Fig. 2 Effects of XCT-790 on the migration and invasion of A549 cells

A: representative images of wound healing at 0, 48, and 72 h in the presence or absence of XCT-790 (1 μmol/L); B: quantitative analysis of wound healing assay for A549 cells treated with XCT-790 (1 μmol/L); C, D: comparison of the number of migrating (C) or invading (D) cells between cells treated with XCT-790 or not (control). Data are presented as $\bar{x}\pm s$ of three independent experiments. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* control

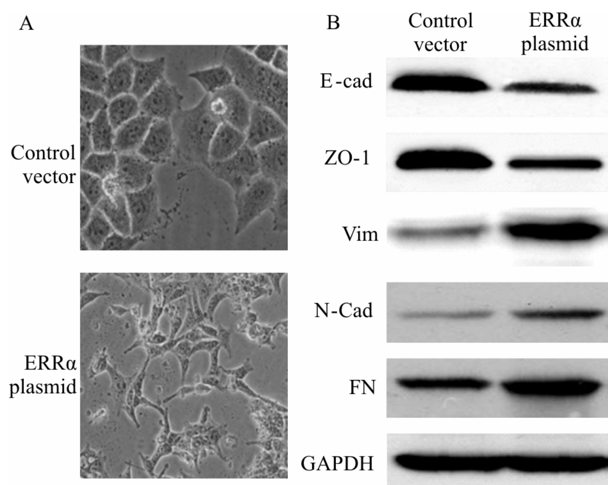

Fig. 3 Over-expression of ERRα-induced EMT of A549 cells

A: cell morphological changes of A549 cells transfected with the ERRα plasmid or the control vector at 48 h detected by phase-contrast microscopy (×200). B: Western blot analysis of epithelial markers E-cad and ZO-1 and mesenchymal makers Vim, FN, and N-Cad in cells transfected with the ERRα plasmid or the control vector at 48 h

2.4 Slug Mediated ERRα-induced EMT of A549 Cells

Transcription factors such as Snail, ZEB1, Twist and Slug have been reported to play an essential role in the progress of EMT[19]. To further investigate the mechanisms by which ERRα induced EMT of A549 cells, the protein and mRNA levels of transcription factors were detected by Western blotting and qRT-PCR, respectively. As shown in fig. 4A, the over-expression of ERRα significantly increased the protein expression of Snail and Slug but Twist and Zeb1. The results of qRT-PCR confirmed that over-expression of ERRα significantly up-regulated the mRNA of Snail and Slug but Twist and Zeb1 (fig. 4B).

To verify whether Snail and Slug are essential for ERRα-induced EMT of A549 cells, cells were transfected with non-targeting control si-RNA, si-Snail, or si-Slug for 24 h, and then transfected with ERRα plasmid for another 48 h. Western blot analysis showed that both Snail and Slug were successfully silenced by their corresponding siRNA (fig. 4C). Further, the biomarkers of EMT were detected by Western blotting. The results showed that Slug but not Snail significantly attenuated the ERRα-induced down-regulation of E-Cad and up-regulation of Vim (fig. 4D). Generally, these results indicated that Slug is essential for ERRα-induced EMT

of lung cancer A549 cells.

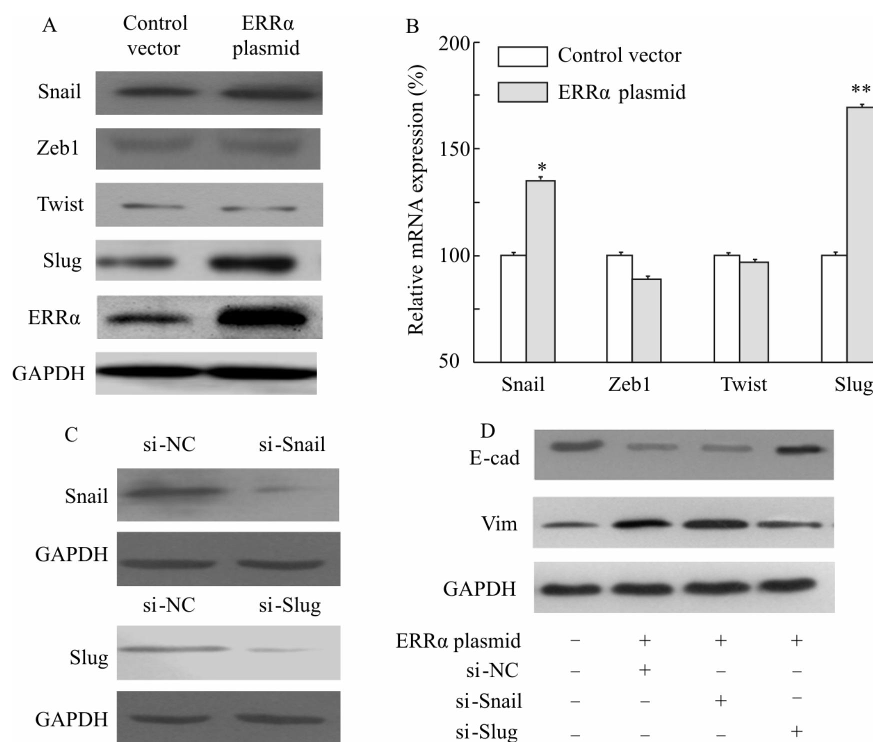

Fig. 4 Slug mediated the ERRα-induced EMT of A549 cells

A: Western blot analysis of the protein expression of transcription factors Snail, Slug, Twist and Zeb1 in cells transfected with ERRα plasmid or the control vector for 24 h; B: comparison of the relative mRNA expression levels of Snail, Slug, Twist and Zeb1; C: Western blot analysis of the protein expression of Snail and Slug in A549 cells transfected with Snail specific si-RNA (si-Snail), Slug specific si-RNA (si-Slug) or negative control si-RNA (si-NC) for 24 h; D: Western blot analysis of the protein expression of E-Cad and Vim in cells transfected with si-Snail, si-Slug, si-NC or ERRα plasmid or not at 48 h

3 DISCUSSION

Several members of the nuclear hormone receptor super family have been reported to be linked directly or indirectly to oncogenesis[20]. ERRα, a member of nuclear receptor super family, which act as a transcription factor, is found to express in various cancer types at the RNA or protein levels[21]. It is over-expressed in tumors of ovarian and mammary origin when compared with the corresponding normal tissue[6]. Furthermore, the over-expression of ERRα is globally associated with a poor prognosis in tumors originating from the endometrium, ovaries, prostate and breasts[22–25]. However, there is a paucity of data about the expression and roles of ERRα in non-estrogen-dependent tumors including lung cancer. To our knowledge, only one study revealed that XCT-790, the inverse agonist of ERRα, can arrest the growth of A549 lung cancer cell via inducing the production of mitochondrial reactive oxygen species (ROS)[26]. The roles of ERRα in the progression of lung cancer cells are yet to be fully elucidated.

The present study revealed that ERRα was highly expressed in lung cancer A549 cells and it could promote the cell proliferation *in vitro*. Correspondingly, the inverse agonist XCT-790 could inhibit the cell proliferation in a concentration-dependent manner. Recent studies revealed that silencing of endogenous ERRα can inhibit the growth of colon cancer cells, and cause a defect in colony formation and *in vivo* tumorigenesis[13]. The promoting effects of ERRα on cell proliferation were also observed in many other cancers such as breast and prostate cancer[24, 25], which might be activated by up-regulating the expression of peroxisome proliferator-activated receptor gamma coactivator-1 beta (PGC-1β) via Her2/IGF-1R signaling pathways and subsequent C-MYC stabilization[12], modulating ROS production[26] and expression of the cell cycle inhibitor $p21^{waf/cip1}$[27].

ERRα has also been reported to modulate the motility of cancer cells[6]. Xu *et al* reported the suppressor of ERRα decreased the migration of breast cancer cells[28]. In the present study, we found that XCT-790 significantly increased wound closure and inhibited the migration and invasion of A549 cells as compared with the control group. It was suggested that inactivation of ERRα can suppress the motility of lung cancer cells. This was consistent with the findings reported by Stein *et al* who revealed that introduction of a small interfering RNA directed to ERRα into the highly aggressive breast carcinoma MDA-MB-231 cell line dramatically reduced the migratory potential of these cells[29].

Recently, ERRα is proved to be a critical positive

regulator of EMT and subsequent CSC-like properties and an inducer of ovarian cancer metastasis, both *in vitro* and *in vivo*[9]. We also observed that the EMT occurred in cells transfected with ERRα plasmid, which was accompanied with down-regulation of epithelial makers E-Cad and ZO-1 and up-regulation of the mesenchymal makers Vim and FN. EMT has been considered to be the first step of tumor invasion and metastasis not only for lung cancer but also for other types of cancer. Therefore, identifying factors that can control EMT and the associated malignant features is critical. Although there is no study concerning the correlation between expression levels of ERRα and clinical outcomes of lung cancer, the positive correlations have been observed in ovarian and breast cancer[11, 25]. Further studies are needed to address the clinical treatment values of ERRα-based targeting therapy for lung cancer.

The molecular mechanism underlying the aggressive nature of EMT remains largely unknown. In the present study, we found that over-expression of ERRα increased the protein and mRNA levels of both Snail and Slug. Meantime, neither mRNA nor protein levels of ZEB1 and Twist were altered. Gene silencing of Slug by si-RNA, but not Snail, successfully abolished the ERRα-induced down-regulation of E-Cad and up-regulation of Vim. Our results suggested that Slug is essential for ERRα-induced EMT of lung cancer A549 cells. Another study revealed that ERRα induced EMT via up-regulation of Snail not only through transcriptional regulation of Snail but also through posttranscriptional regulation[9], which is supported by increasing evidence that members of the Snail family of transcription factors can be differentially regulated[30]. Moreover, other transcription factors such as ZEB2 and E47, which have been reported to control the EMT during cancer development[19], should be further studied to investigate whether they participate in the ERRα-induced EMT.

In summary, our study demonstrated for the first time that ERRα is a critical positive regulator of EMT of lung cancer cells. Moreover, we revealed that Slug, but not other transcription factors, mediated the ERRα-induced EMT. Given that EMT is a fundamentally important process for the metastasis of lung cancer cells *in vivo*, the clinical correlation of ERRα and lung cancer progression should be further investigated.

Conflict of Interest Statement

The authors declare no conflict of interest.

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