Estrogen-related receptor γ promotes the migration and metastasis of endometrial cancer cells by targeting S100A4

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Abstract. S100 calcium binding protein A4 (S100A4) is a well-established tumor metastasis mediator in various malignancies, including endometrial cancer (EC). However, the regulatory mechanism underlying S100A4 expression remains elusive. In the present study, by analyzing public datasets and clinical samples, we found that estrogen-related receptor γ (ERRγ) was upregulated and positively correlated with S100A4 transcription in EC. ERRγ knockdown inhibited S100A4 expression and promoted the expression of its downstream target E-cadherin, and vice versa. Mechanistic studies indicated that ERRγ enhanced the promoter activity of S100A4 to facilitate its transcription. In addition, knockdown of ERRγ suppressed migration and invasion of EC cells *in vitro*, while ectopic ERRγ expression promoted migration and invasion of EC cells *in vitro* and tumor growth *in vivo*. Importantly, restoration of S100A4 expression prevented EC cells from undergoing ERRγ-mediated changes in these biological features. In addition, synchronous changes in S100A4 and ERRγ expression were observed after incubation with estrogen. Overall, ERRγ may exert oncogenic activity mainly associated with aggressiveness of EC by activating S100A4 transcription and thus may be a novel therapeutic target in EC.

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Introduction

Endometrial cancer (EC) is the most common gynecologic tumor in developed countries, with an increasing prevalence (1). It is estimated that 61,380 new cases and 10,920 deaths resulting from EC occurred in 2017 (2). Adenocarcinoma of the endometrium accounts for over 70% of all uterine cancers. Most patients (80%) are diagnosed with early disease and can be surgically cured. However, the outcome of advanced or recurrent cases remains far worse, and the adjuvant treatment options are quite limited (1). Discovery of novel targets is warranted to better understand the EC pathogenesis and to develop new therapeutic approaches for this disease. It is well known that tumor progression and metastasis are often linked to epithelial-mesenchymal transition (EMT). During this process, a more invasive cell phenotype is established, accompanied by alterations in the expression of many core molecules, particularly E-cadherin (3). Thus, it is worthwhile to explore the regulatory mechanism of EMT in the tumor biology of EC.

S100 calcium binding protein A4 (S100A4) has been shown to be involved in biological functions that contribute to malignant tumors, such as proliferation, apoptosis, metastasis, angiogenesis and immune evasion (4). More importantly, S100A4 plays pivotal roles in tumor invasion by triggering EMT, mechanically serves as a downstream target gene of the wnt/β-catenin pathway, modulates membrane integrin signaling, and directly promotes cell motility through interaction with cytoskeletal proteins such as myosin, actin and tropomyosin (5-7). Elevated S100A4 expression has been found in many types of tumors and is closely related to poor outcome in tumor patients (8). Our previous research revealed that S100A4 is highly expressed in EC cells, and knockdown of S100A4 expression resulted in suppression of the migration and invasion capability of EC cells, which may partially occur via EMT-related modifications (9). However, the regulatory mechanisms of S100A4 expression in EC remain to be elucidated.

Estrogen-related receptors (ERRs; ERRα, ERRβ and ERRγ) comprise a subgroup of orphan nuclear receptors that share highly homologous DNA-binding domains with

estrogen receptors. However, ERRs do not bind to endogenous estrogens or their derivatives, and thus are designated orphan receptors (10). High ERRγ expression levels are often associated with high metabolic demand in human tissues, such as skeletal muscle, heart and brown adipose tissue. Accumulating evidence indicates a central role of ERRγ in metabolic genes and cellular energy metabolism regulation (11). Apart from metabolic disease, recent studies have revealed the clinical significance of ERRγ in several cancer types, including EC. In breast cancer, ERRγ is generally overexpressed and related to lymph node status and upregulated during tamoxifen resistance acquisition, indicating a cancer promoting role of ERRγ (12,13). The role of ERRγ in EC remains unclear. Overexpression of ERRγ is correlated with increased clinical stage, deeper myometrium invasion and positive lymph node status (14,15). In addition, ERRγ mediates estrogen-induced proliferation of EC cells (16). However, the effect of ERRγ on EC cell migration and metastasis has never been explored.

In the present study, augmented expression of ERRγ was found, and for the first time, a correlation between ERRγ and S100A4 expression was identified in clinical EC tissues via experimental techniques and public database mining. Furthermore, ERRγ directly facilitates S100A4 transcription through promoter activation, thus promoting migration and invasion of EC cells both *in vitro* and *in vivo*, demonstrating the emerging roles of ERRγ in EC progression through transcriptional regulation of S100A4.

Materials and methods

Patients and specimens. The present study was performed in accordance with the Declaration of Helsinki, and approval to conduct the present study was obtained from the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (IORG no. IORG0003571). Tissues were collected after receiving informed consent from the patients. Formalin-fixed paraffin-embedded specimens were obtained from 20 primary EC patients [age (mean ± SD), 51.5±12.1 years] who had undergone surgical resection at WuHan Union Hospital between September 2015 and December 2016. Those that had already received adjuvant therapy, such as chemotherapy, hormone therapy or radiotherapy, were excluded. Fresh specimens from the above-mentioned EC patients and another 20 normal endometrium cases (age, 49.6±9.4 years) were collected for protein and total RNA extraction and stored in liquid nitrogen until use.

Immunohistochemical staining. Fresh specimens were fixed in 10% formaldehyde for at least 24 h and embedded in paraffin. Tissue slides with 4-μm-thick sections were constructed and dewaxed in xylene and rehydrated in a graded alcohol series. Antigen retrieval was conducted by heating slides in 0.01 M of sodium citrate buffer for 20 min. Endogenous non-specific peroxidase activity was blocked with 3% H_2O_2 for 15 min, and non-specific staining was blocked by incubation with 10% normal goat serum for 30 min. Then, the samples were incubated with 200 μl of primary antibodies against ERRγ (1:400; cat. no. ab49129; Abcam, Cambridge, MA, USA), S100A4 (1:400; cat. no. 13018S; Cell Signaling Technology, Inc., Beverly, MA, USA) and Ki-67 (1:200; cat. no. sc-23900; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) at 4˚C overnight. After being washed with phosphate-buffered saline (PBS), the slides were incubated with EnVision/HRP, rabbit secondary antibody (1:1,000; cat. no. GB23303; Servicebio Technology, Co., Ltd., Wuhan, China) for 30 min. Diaminobenzidine substrate was used for visualization, followed by counterstaining with hematoxylin. Finally, the slides were dehydrated and mounted. The immunohistochemistry scoring strategy was performed as previously described (9).

Western blot analysis. Collected fresh EC tissues or cultured EC cells were washed with ice-cold PBS 3 times and lysed with radio-immunoprecipitation assay buffer containing protease inhibitors. The protein concentration was determined using a bicinchoninic acid (BCA) protein assay kit. Equal amounts of proteins (30 μg) were added to 12% SDS-PAGE gels and transferred onto nitrocellulose membranes. After being blocked with 5% skim milk in 1X TBS buffer containing 0.1% Tween-20 at room temperature for 1 h, the membranes were incubated with primary antibodies against ERRγ (1:800; cat. no. ab49129; Abcam), S100A4 (1:800; cat. no. 13018S; Cell Signaling Technology), GAPDH (1:1,000; cat. no. sc-66163; Santa Cruz Biotechnology) and E-cadherin (1:200; cat. no. sc-52327; Santa Cruz Biotechnology) at 4˚C overnight. The target proteins were visualized using enhanced chemiluminescence reagents (Thermo Fisher Scientific, Inc., Waltham, MA, USA) after incubation with goat anti-rabbit secondary antibody (1:5,000; cat. no. sc-2004; Santa Cruz Biotechnology). Enhanced chemiluminescence reagents (Thermo Fisher Scientific) were used for bands detection. The optical density was quantified using Bio-Rad Image Lab™ v4.1 software.

Real-time quantitative RT-PCR. Total RNA from EC tissues and cultured EC cells was isolated with RNAiso Plus (Takara Bio, Co., Ltd., Otsu, Japan) following the manufacturer's protocol. The reverse transcription reactions were carried out using a PrimeScript RT reagent kit (Takara Bio). The primers involved were as follows: ERRγ (102 bp), 5'-CCCGACAGT GACATCAAAGCC-3' (sense) and 5'-CGTGGAGAAGCC TGGAATATGC-3' (antisense); S100A4 (251 bp), 5'-TACTCG GGCAAAGAGGGTGA-3' (sense) and 5'-CATTTCTTCCTG GGCTGCTTA-3' (antisense); E-cadherin (162 bp), 5'-GAG AACGCATTGCCACATACAC-3' (sense) and 5'-GAGCAC CTTCCATGACAGACCC-3' (antisense); GAPDH (255 bp), 5'-ACTTTGGTATCGTGGAAGGACTAT-3' (sense) and 5'-GTTTTTCTAGACGGCAGGTCAGG-3' (antisense). Real-time PCR was conducted with Premix Ex Taq (Takara Bio), as previously described (9). The $2^{-\Delta\Delta Ct}$ method was employed for relative transcript abundance determination. Each experiment was performed in triplicate at least 3 times.

Cell culture and transfection. The human EC cell lines Ishikawa, AN3CA, HEC-1A and HEC-1B were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in appropriate medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml of penicillin and 100 μg/ml of streptomycin. All the cells were incubated at 37˚C in a humidified atmosphere containing 5% CO_2.

Table I. Oligonucleotide sets used for constructs and short hairpin RNAs.

Oligo set	Sequences
sh-Scb	5'-CCGGTTCTCCGAACGTGTCACGTTTCAAGAGAACGTGACACGTTCGGAGAATTTTTG-3' (sense)
	5'-AATTCAAAAATTCTCCGAACGTGTCACGTTCTCTTGAAACGTGACACGTTCGGAGAA-3' (antisense)
sh-ERRγ-1	5'-CCGGCCTCACTACACTGTGTGACTTCTCGAGAAGTCACACAGTGTAGTGAGGTTTTTG-3' (sense)
	5'-AATTCAAAAACCTCACTACACTGTGTGACTTCTCGAGAAGTCACACAGTGTAGTGAGG-3' (antisense)
sh-ERRγ-2	5'-CCGGCGAATGAATGTGAAATCACAACTCGAGTTGTGATTTCACATTCATTCGTTTTTG-3' (sense)
	5'-AATTCAAAAACGAATGAATGTGAAATCACAACTCGAGTTGTGATTTCACATTCATTCG-3' (antisense)
sh-S100A4	5'-CCGGCGCCATGATGTGTAACGAATTCTCGAGAATTCGTTACACATCATGGCGTTTTTG-3' (sense)
	5'-AATTCAAAAACGCCATGATGTGTAACGAATTCTCGAGAATTCGTTACACATCATGGCG-3' (antisense)

ERRγ, estrogen-related receptor γ; S100A4, S100 calcium binding protein A4; sh-Scb, scramble short hairpin RNA.

Plasmid/construct transfection. Oligonucleotides encoding short hairpin RNAs (shRNAs) for ERRγ (sh-ERRγ), S100A4 (sh-S100A4), scramble shRNA (sh-Scb), and S100A4 expression cDNA (GV230-S100A4) were purchased from Shanghai GeneChem (Shanghai, China). Invitrogen™ Lipofectamine 2000 (Thermo Fisher Scientific) was used for transfection. The target sequences for synthetic oligonucleotide primers are listed in Table I. A lentiviral vector carrying ERRγ gene fragments was constructed to upregulate ERRγ expression in HEC-1A and AN3CA cells. The empty vector served as the control. Stable cell lines were selected by administration of geneticin or puromycin (Sigma-Aldrich; Merck KGaA).

Luciferase reporter assay. Sequences containing different S100A4 promoter sections were cloned and sub-cloned to pcDNA3.0 basic vectors. EC cells were plated on 24-well plates overnight and co-transfected with luciferase reporter vectors and the internal control plasmid pRL-SV40 (Promega Corp., Madison, WI, USA) carrying the *Renilla* luciferase gene for 24 h. Firefly and *Renilla* luciferase activities were measured with a dual-luciferase assay system kit (Promega Corp.). Experiments performed for the analysis were repeated at least 3 times.

Transwell migration and invasion assays. Migration assays were performed using Transwell inserts with 8.0-μm pore membrane filters (Corning Costar, Tewksbury, MA, USA). For invasion assays, the microfilters were precoated with 50 μl of Matrigel matrix (BD Biosciences, Sparks, MD, USA). Then, $5x10^4$ homogeneous single cells were plated in the upper chambers of each well with low-serum medium (1% FBS), and a chemo-attractant (medium containing 10% FBS) was added to the lower chamber, followed by a 24-h incubation. Afterwards, the membranes were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet. The numbers of migrating and invading cells were determined in 5 random fields of each membrane using CX23 Olympus light microscopy (Olympus Optical Co., Ltd., Tokyo, Japan). Three replicates were performed for this analysis.

In vivo tumor growth assay. All animal experiments were approved by the Animal Care Committee of Tongji Medical College. Female 4- to 6-week-old and weight ~20 g BALB/c nude mice were purchased from Vital River Laboratory Animal Technology Co., Ltd. (Beijing, China). The animals were housed at 23±1˚C under a standard 12h/12h light-dark cycle with free access to food and water. Mice were injected subcutaneously in the right flank with $5x10^6$ HEC-1A cells stably transfected with ERRγ or empty vectors as indicated (n=5 per group). Tumor volume (V) was measured every 4 days and calculated via the formula: V = length x width^2/2. At 42 days after injection, the mice were sacrificed, and subcutaneous tumors were weighed and photographed using a camera. The protein levels of ERRγ, S100A4 and Ki-67 were analyzed in tumor tissues via immunohistochemistry.

Statistical analysis. Statistical analysis was performed using GraphPad Prism v5.0 statistical software (GraphPad Software, Inc., La Jolla, CA, USA). All results are expressed as the mean ± standard error of the mean (SEM). Differences among groups were determined with Student's t-test or one-way analysis of variance (ANOVA). The post hoc test was performed for intergroup comparison of parametric data. Pearson's coefficient correlation was applied to investigate the association between ERRγ and S100A4 expression levels. P-values <0.05 were statistically significant.

Results

ERRγ is highly expressed and positively correlated with S100A4 expression in EC tissues. Mining the publicly available databases Gene Expression Omnibus website (GEO; http://www.ncbi.nlm.nih.gov/geo/) and R2, microarray analysis and visualization platform (http://hgserver1.amc.nl/cgi-bin/r2/main.cgi) revealed increased ERRγ transcription levels in EC compared with benign endometrium (Fig. 1A), and an inverse correlation between ERRγ and S100A4 transcription levels was observed in several types of cancers, including non-small cell lung and colon cancer, squamous cell carcinoma of the tongue, oral cavity cancer, and Wilms' tumors (data not shown). To investigate ERRγ expression in EC, fresh tissues from 20 well established primary EC patients and 20 normal endometrium cases were collected. Real-time quantitative RT-PCR and western blotting showed higher

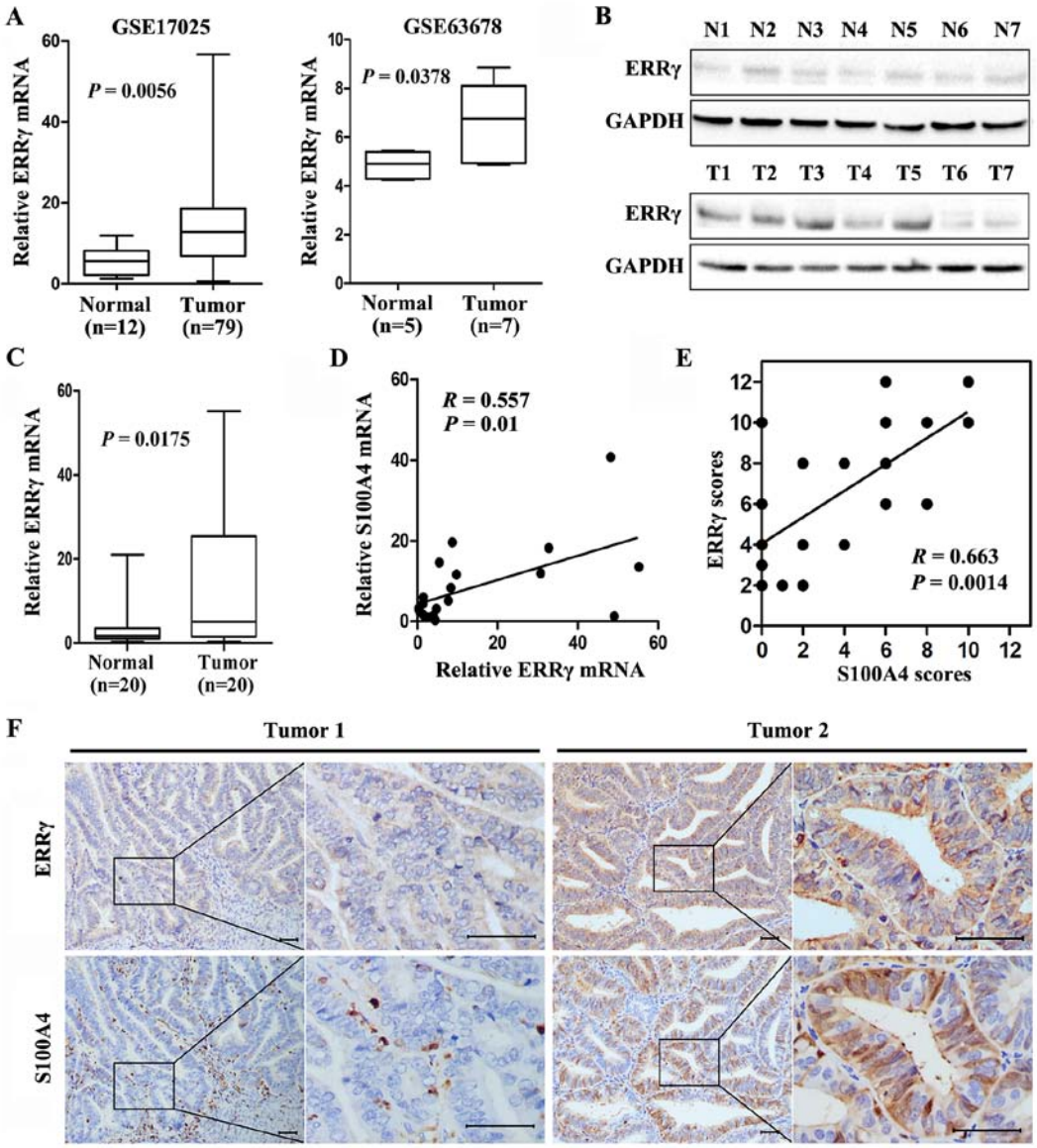

Figure 1. ERRγ is highly expressed and positively correlated with S100A4 expression in EC tissues. (A) Transcription levels of ERRγ in endometrial cancer (tumor) and benign endometrium (normal) tissues from GEO databases (GSE17025 and GSE63678) (38,39). (B) Representative western blots of ERRγ protein from normal endometrium (N) and endometrial cancer (T). (C) The mRNA levels of ERRγ in endometrial cancer (tumor) and benign endometrium (normal) tissues as indicated by RT-qPCR. (D) Correlation of ERRγ and S100A4 transcription levels in endometrial cancer patients. (E) Positive correlation between the ERRγ and S100A4 staining scores in EC tissues. (F) Representative immunohistochemical staining of ERRγ and S100A4 (brown) in EC tissues. Scale bars, 50 μm. S100A4, S100 calcium binding protein A4; ERRγ, estrogen-related receptor γ.

expression levels of ERRγ in EC specimens than the levels noted in the benign endometrium (Fig. 1B and C). In addition, a positive correlation between ERRγ and S100A4 transcription levels in EC tissues was verified with real-time quantitative RT-PCR (correlation coefficient R=0.557, P=0.01, Fig. 1D) and immunohistochemical staining (correlation coefficient R=0.663, P=0.0014, Fig. 1E and F). These results indicated that ERRγ is overexpressed and positively correlated with S100A4 in EC patients.

ERRγ transcriptionally regulates the expression of S100A4 in cultured EC cells. The transcription levels of ERRγ are low in most EC cell lines, as indicated by the Cancer Cell Line Encyclopedia (CCLE) program (http://www.broadinstitute.org/ccle; Fig. 2A), and this finding was validated by western blotting and real-time quantitative RT-PCR in 4 representative EC cell lines (Fig. 2B and C). Expression of ERRγ was relatively high in HEC-1B cells but almost undetectable in Ishikawa, HEC-1A and AN3CA EC cells. To explore the hypothesis that ERRγ may modulate the expression of S100A4 in EC, HEC-1B cells were stably transfected with sh-ERRγ, leading to decreased protein and transcription levels of ERRγ and S100A4 compared to those in cells transfected with sh-Scb (Fig. 2D and E). Additionally, expression of the S100A4 downstream gene E-cadherin was significantly upregulated in ERRγ-silenced EC cells. As restoration of S100A4 partially abolished the impact of ERRγ on E-cadherin expression (data not shown), we believed that ERRγ regulated E-cadherin expression through S100A4 in EC cells. Inversely, stable transfection of HEC-1A and AN3CA EC cells with ERRγ notably upregulated and downregulated the expression of S100A4 and E-cadherin, respectively, compared with

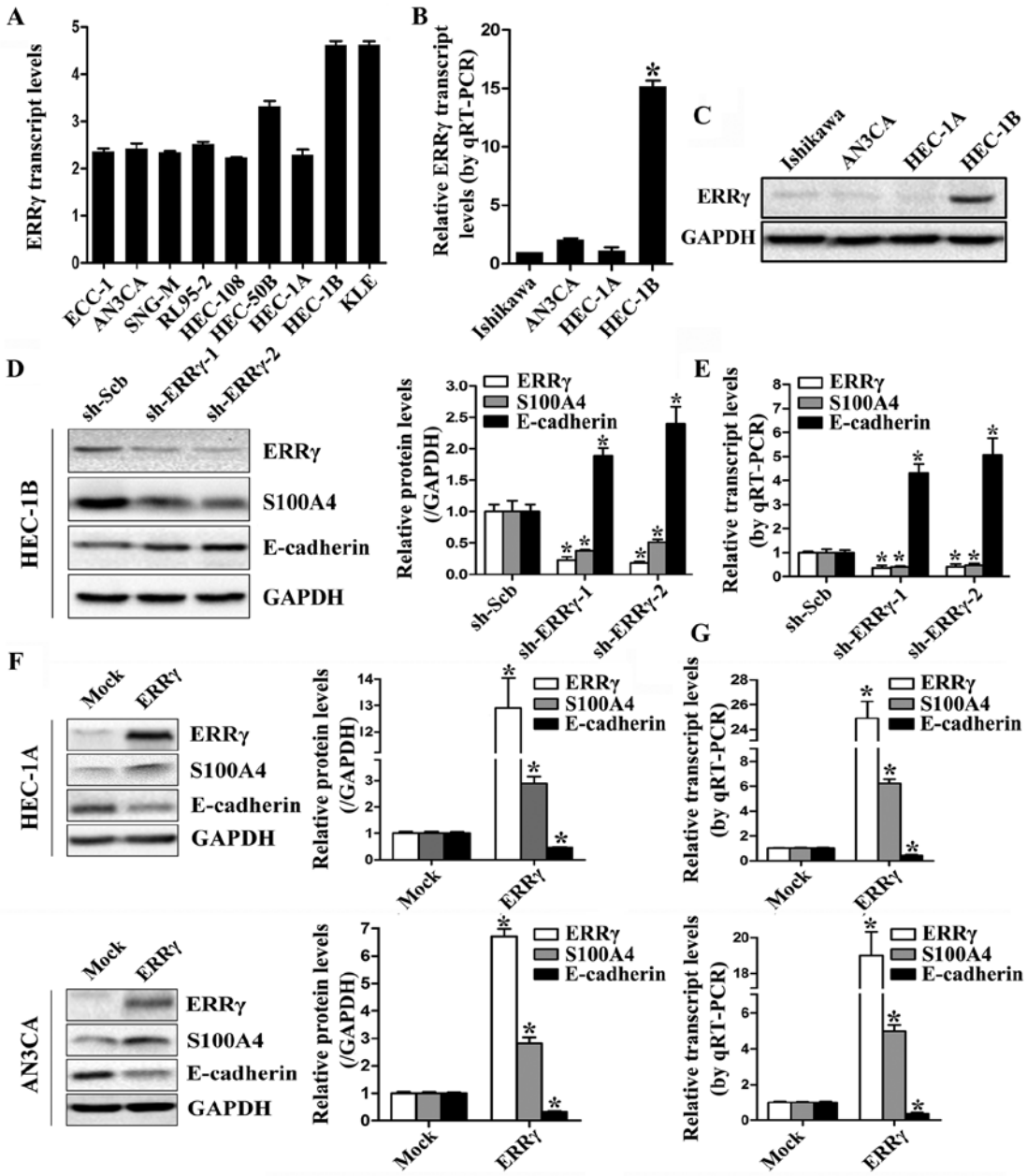

Figure 2. ERRγ regulates the expression of S100A4 in cultured EC cell lines. (A) Transcription levels of ERRγ in 9 EC cell lines extracted from the Cancer Cell Line Encyclopedia (CCLE) program (http://www.broadinstitute.org/ccle). (B and C) qRT-PCR and western blotting showing the mRNA and protein levels of ERRγ in Ishikawa, AN3CA, HEC-1A and HEC-1B cells. (D and E) Western blotting and qRT-PCR results indicating the ERRγ, S100A4 and E-cadherin protein and mRNA levels in HEC-1B cells transfected with scramble short hairpin RNA (sh-Scb) or sh-ERRγ. (F and G) Western blotting and qRT-PCR results indicating the ERRγ, S100A4 and E-cadherin protein and mRNA levels in HEC-1A (top) and AN3CA (bottom) cells transfected with empty vector (mock) and ERRγ. *P<0.05. S100A4, S100 calcium binding protein A4; ERRγ, estrogen-related receptor γ.

cells transfected with the empty vector (Fig. 2F and G). These results demonstrated that ERRγ could modulate the expression of S100A4 in EC cells.

To investigate whether ERRγ could transcriptionally increase S100A4 expression, computational assessment from the JASPAR CORE Database (http://jaspar.genereg.net) revealed three potential binding sites of ERRγ within the S100A4 promoter, located 368-377, 639-648 and 731-740 bp upstream of the transcription start site (TSS). An S100A4 promoter luciferase reporter and its truncation vectors were constructed and used to transfect EC cells. A dual-luciferase assay revealed that the region -656/-784 bp relative to the TSS was essential for S100A4 promoter activities, and deletion of this region resulted in remarkably decreased S100A4 promoter activities in cultured HEC-1A and AN3CA cells (Fig. 3A). Moreover, knockdown of ERRγ in cultured HEC-1B cells attenuated the promoter activities of S100A4, and ectopic expression of ERRγ enhanced the S100A4 promoter activities in HEC-1A and AN3CA cells (Fig. 3B and C). These results indicated that ERRγ could trigger S100A4 transcription through promoter activation.

ERRγ modulates the migration and invasion capability of EC cells through S100A4 in vitro. We first explored the effects of

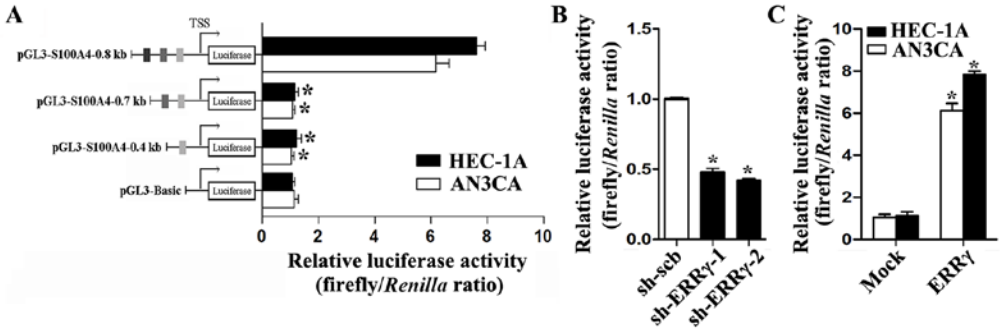

Figure 3. ERRγ transcriptionally activates S100A4 expression in cultured EC cells. (A) Dual-luciferase assay showing the S100A4 promoter activities with different truncations in HEC-1A and AN3CA cells. (B) Dual-luciferase assay displaying the promoter S100A4 activities in HEC-1B cells transfected with sh-Scb or sh-ERRγ. (C) Dual-luciferase assay showing the S100A4 promoter activities in HEC-1A and AN3CA cells transfected with mock or ERRγ. *P<0.05. S100A4, S100 calcium binding protein A4; ERRγ, estrogen-related receptor γ.

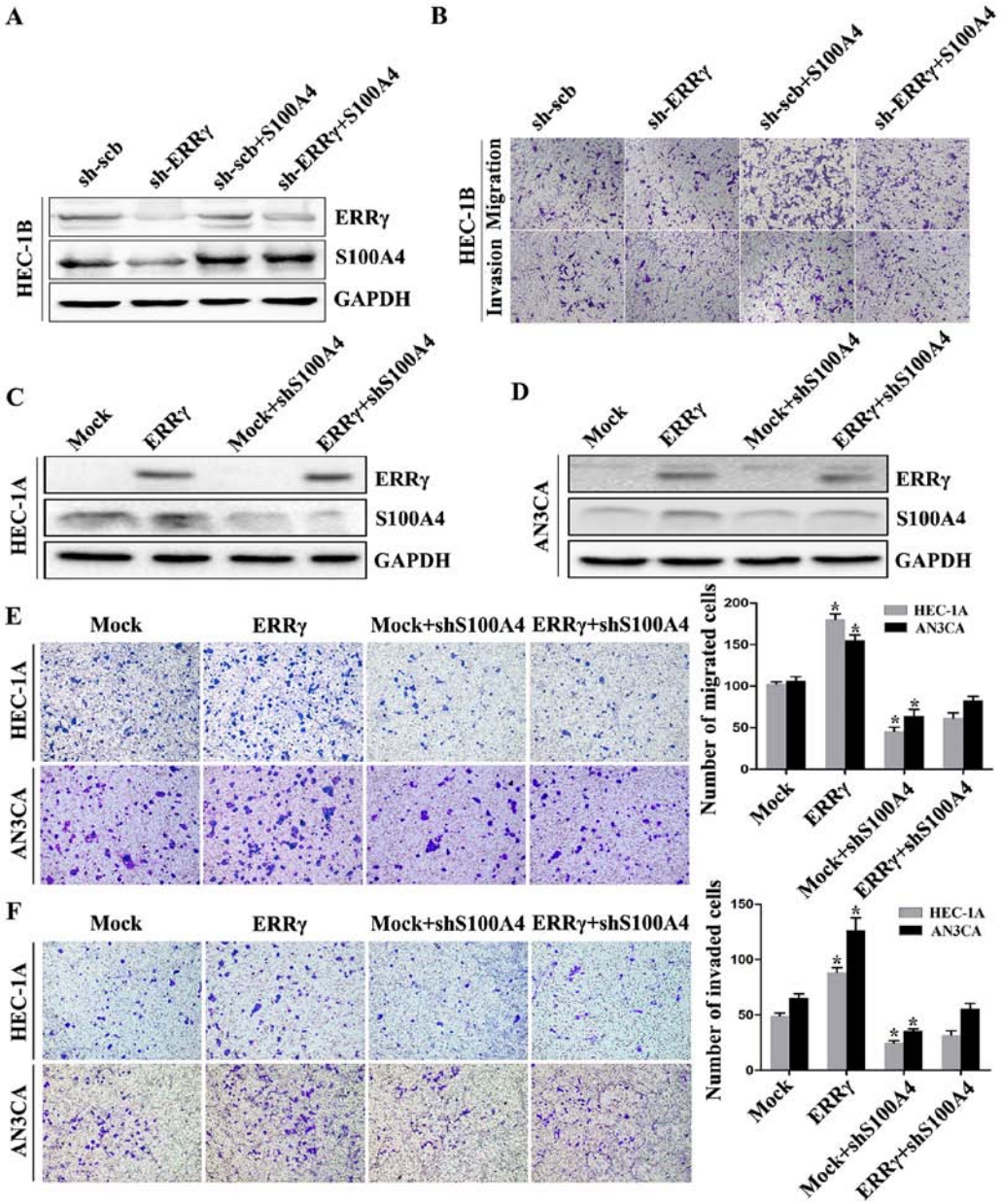

Figure 4. ERRγ modulates the migration and invasion capability of EC cells through S100A4 *in vitro*. (A) Western blotting showing the protein levels of ERRγ and S100A4 in EC cells stably transfected with sh-Scb, sh-ERRγ or co-transfected with S100A4 expression vector. (B) Representation of migrated and invaded HEC-1B cells upon transfection with sh-Scb, sh-ERRγ or co-transfected with S100A4 revealed with Transwell assays after 24 h. (C and D) Western blotting showing the protein levels of ERRγ and S100A4 in EC cells stably transfected with empty vector (mock), ERRγ and co-transfected with sh-S100A4. (E and F) Representation (left) and quantification (right) of migrated and invaded HEC-1A and AN3CA cells upon transfection with mock, ERRγ, or co-transfection with sh-S100A4 determined with Transwell assays after 24 h. *P<0.01. S100A4, S100 calcium binding protein A4; ERRγ, estrogen-related receptor γ.

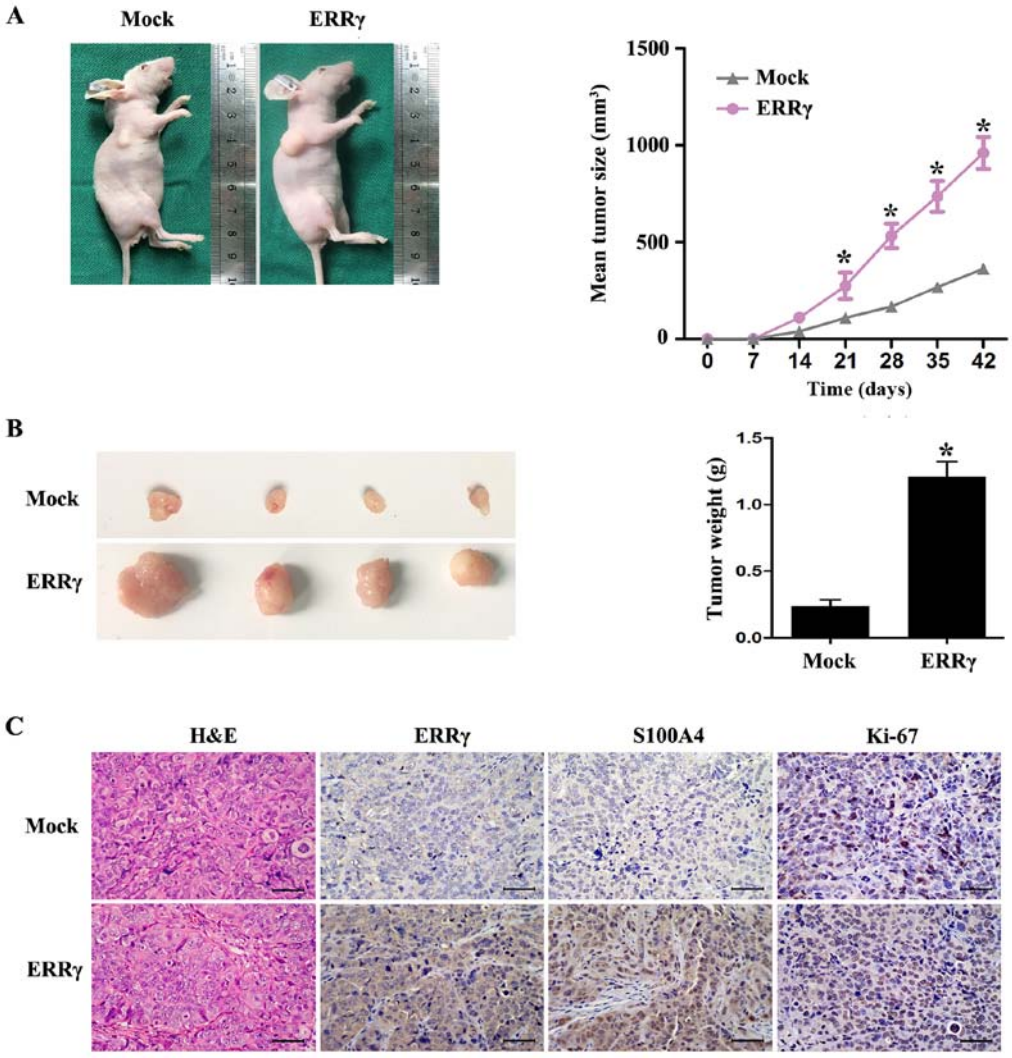

Figure 5. ERRγ promotes the growth of EC cells *in vivo*. (A) Tumor growth curve of HEC-1A cells ($5x10^6$) stably transfected with ERRγ or empty vector (mock) injected into athymic nude mice (n=5 for each group). (B) Representation (left) and quantification (right) of xenograft tumors formed by injection of HEC-1A cells stably transfected with mock or ERRγ. (C) The hematoxylin and eosin and immunohistochemical analyses of xenografts. ERRγ, S100A4 and Ki-67 expression were verified in the mock and intervention groups. Scale bars, 50 μm. *P<0.01. S100A4, S100 calcium binding protein A4; ERRγ, estrogen-related receptor γ.

ERRγ knockdown and S100A4 restoration on the migration and invasion capacity of EC cells. ERRγ knockdown decreased the expression of S100A4, and ectopic expression of S100A4 restored the ERRγ knockdown-induced S100A4 downregulation in HEC-1B cells (Fig. 4A). In Transwell migration assays, ERRγ knockdown inhibited the migration capability of HEC-1B cells compared to that of cells transfected with sh-Scb. Matrigel invasion assays showed that HEC-1B cells stably transfected with sh-ERRγ presented an impaired invasion capacity compared to sh-Scb group cells. In addition, restoration of S100A4 expression rescued the EC cells from the defects in migration and invasion capabilities induced by ERRγ downregulation (Fig. 4B). These results revealed that S100A4 was involved in ERRγ knockdown-induced EC cell migration and invasion inhibition.

The impacts of ERRγ overexpression and S100A4 restoration on cultured EC cells were further studied. Transfection of HEC-1A and AN3CA cells with sh-S100A4 resulted in reduced S100A4 protein levels and restored the upregulation of S100A4 induced by ERRγ (Fig. 4C and D). In Transwell migration assays, ectopic ERRγ expression increased the migration capability of HEC-1A and AN3CA cells compared with cells transfected with empty vector (mock) (Fig. 4E). Matrigel invasion assays revealed that EC cells stably transfected with ERRγ exhibited an enhanced invasion capacity compared with mock group cells (Fig. 4F). Moreover, restoration of S100A4 expression prevented the enhanced migration and invasion capacity in EC cells induced by stable overexpression of ERRγ (Fig. 4E and F). These findings suggest that S100A4 could, at least in part, mediate ERRγ-induced promotion of EC cell aggressiveness.

ERRγ promotes the growth of EC cells in vivo. The efficacy of ERRγ overexpression on tumor growth *in vivo* was further investigated. HEC-1A cells with fixed ERRγ expression were subcutaneously injected into nude mice, leading to an increased proliferative index and tumor weight compared with tumors formed from cells transfected with the empty vector (Fig. 5A and B). Immunohistochemical analysis also showed that the expression of ERRγ and its downstream gene

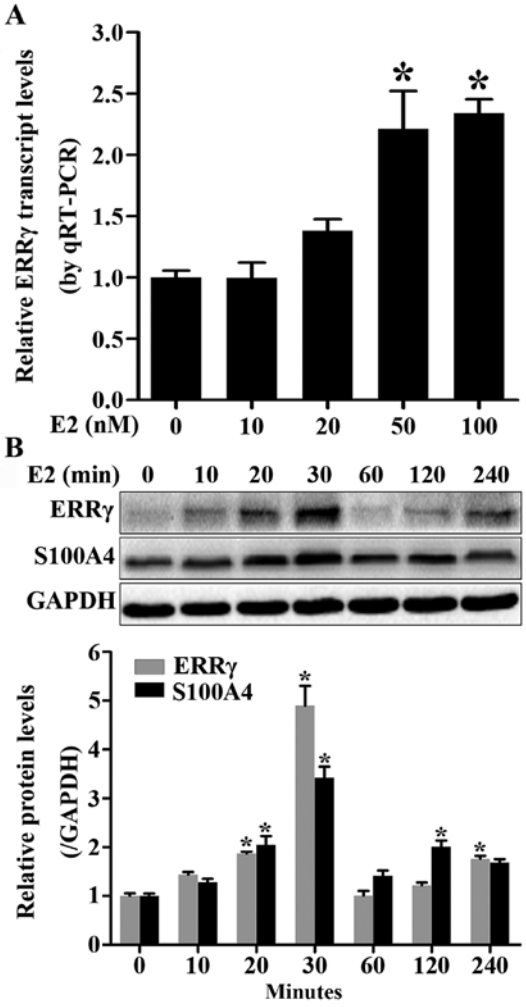

Figure 6. Upregulation of ERRγ and S100A4 in estrogen-treated HEC-1A cells. (A) Transcription levels of ERRγ in HEC-1A cells treated with 0, 10, 20, 50 and 100 nM of estrogen for 24 h. (B) Time course of ERRγ and S100A4 protein and mRNA levels in HEC-1A cells treated with 50 nM of estrogen for 0, 10, 20, 30, 60, 120 and 240 min. *P<0.01. S100A4, S100 calcium binding protein A4; ERRγ, estrogen-related receptor γ.

S100A4 were increased by stable transfection with ERRγ. Notably, the cell proliferation marker Ki-67 was also upregulated in HEC-1A cells (Fig. 5C). These results revealed an oncogenic role of ERRγ in EC and were consistent with the *in vitro* studies.

ERRγ and S100A4 are upregulated in estrogen-treated HEC-1A cells. Since estrogen is a major factor in EC pathogenesis and progression, we hypothesized that estrogen may affect the expression of ERRγ and S100A4 in EC cells. We first manipulated HEC-1A cells with increasing estrogen concentrations (0, 10, 20, 50 and 100 nM) for 24 h. In real-time quantitative RT-PCR assays, ERRγ mRNA was significantly increased when estrogen concentration reached 50 nM, and no differences were detected between the 50- and 100-nM groups (Fig. 6A). We next treated HEC-1A cells with 50 nM of estrogen for increasing durations (0, 10, 20, 30, 60, 120 and 240 min). Western blot analysis revealed that there was a time-dependent diversification in the protein levels of both ERRγ and S100A4, and both reached a peak at 30 min (Fig. 6B). Furthermore, S100A4 levels increased almost coincidently with ERRγ, and a positive correlation was detected (correlation coefficient R=0.886, P=0.0079). Mining the public GEO database (GSE11869) revealed that a positive correlation between ERRγ and S100A4 expression has also been found elsewhere in several types of EC cells after estrogen stimulation (correlation coefficient R=0.448, P<0.0001). The above findings suggest that estrogen may be an upstream regulator of ERRγ and S100A4 expression in EC.

Discussion

It is generally accepted that S100A4 has profound impacts on numerous types of cancers, including EC, and S100A4 upregulation results in tumor progression and aggressiveness. Additionally, overexpression of S100A4 is a predictive indicator of metastasis and poor survival of cancer patients (17-20). Our previous studies demonstrated that S100A4 promoted endometrial cancer (EC) cell aggressiveness via EMT-related modifications (9). However, the regulatory mechanisms essential for S100A4 expression in EC remain largely unknown. Studies have suggested that tyrosine-protein kinase erbB 2 (ERBB2) receptor signaling and integrin signaling regulate S100A4 expression in human medulloblastoma and breast cancer cells (21,22). More importantly, S100A4 gene expression can be regulated at the transcriptional level, because its promoter contains several putative regulatory elements for transcription factors. In colorectal cancer (CRC), functionally active β-catenin is indispensable for induction of S100A4 expression and results in enhanced S100A4-induced migration and invasion (23). An electrophoretic mobility shift assay (EMSA) and chromatin immunoprecipitation (ChIP) assay further confirmed binding of β-catenin to the S100A4 promoter (7). In the present study, synthetic approaches were employed to analyze transcription profiling of several cancer specimens and transcription factor binding reported in public databases and identified ERRγ as a crucial modulator facilitating S100A4 expression in EC. Notably, ERRγ is highly expressed and positively correlated with S100A4 levels in several types of cancers, including EC specimens.

Early works regarding ERRγ and malignancy mainly focused on the potential crosstalk of ERRγ with the classical estrogen pathway and excavating its master regulation role in energy metabolism (24-26). ERRγ may exert oncogenic or tumor suppressive functions with tumor specificity. High ERRγ expression is correlated with more favorable clinical outcomes in ovarian, breast, and prostate cancer, indicating its tumor-suppressing function in these cancers (13,27,28). Conversely, ERRγ-positive staining in hepatocellular carcinoma (HCC) specimens was remarkably higher than that in adjacent non-tumor liver tissues and was associated with advanced clinical stage and pathological grade, and knocking down ERRγ inhibited HCC cell proliferation and induced G1-phase arrest (29). In human EC, ERRγ is expressed in ~31.3% of EC tissues, and its immunoreactivity was correlated with worse progression-free survival and overall survival. Interestingly, the opposite EC cell responsiveness was observed under forced ERRγ expression or estrogen stimulation with ERα status dependence (15). In addition, the transcription levels of ERRγ in EC were increased with clinical staging, myometrial invasion, and metastatic lymph nodes. Inhibition

of ERRγ activity attenuated estrogen-induced proliferation of EC cells through AKT and ERK1/2 signaling abolition (16). However, the exact biological functions of ERRγ in EC have never been explored. By means of gain- and loss-of-function studies, we demonstrated that ERRγ facilitated migration and invasion of EC cells *in vitro* and promoted tumor growth *in vivo*, suggesting an oncogenic role of ERRγ during EC progression.

Strict binding site specificity experiments indicated that the 3 members of ERRs preferentially recognize almost identical DNA elements, distinct from the traditional estrogen receptor element (ERE), referred to as the estrogen-related receptor response element (ERRE; TnAAGGTCA) (10). Subsequent studies identified widespread distribution of ERRγ targets, and transcriptionally active ERRγ forms a heterodimer or homodimer that binds to the promoter of target genes, while ligand is unnecessary for ERRγ activity (30,31). Studies have confirmed that ERRγ regulates target genes mainly involved in cellular metabolism, including tricarboxylic acid (TCA) cycle genes, fatty acid β-oxidation (FAO) genes, and electron transport chain (ETC) genes (32). Ectopic expression of ERRγ enhanced oxidative phosphorylation in breast cancer cells, and the shift to oxidative metabolism attenuated breast cancer cell proliferation and tumor growth *in vitro* and *in vivo* (33). In addition, ERRγ can also directly bind to genes involved in cell growth, such as p21 and p27. Consistent with the favorable role of ERRγ in breast cancer, ERRγ reprograms the genetic profiles of breast cancer cells in a manner characteristic of mesenchymal-to-epithelial transition, in which E-cadherin was activated by ERRγ directly (34). The target genes of ERRγ involved in initiation and aggressiveness of EC still warrant investigation. In the present study, we showed that ERRγ facilitated transcription of S100A4 in EC cells via S100A4 promotor activation. Furthermore, since restoration of S100A4 expression rescued EC cells from ERRγ-induced phenotype changes in aggressiveness, ERRγ may exert its oncogenic functions by activating S100A4 transcription in EC.

Estrogen is not a natural ligand for ERRγ, as indicated by ligand binding studies and transfection experiments with reporter genes. However, ERRγ stimulates ERE-mediated transcription and functions as an estrogen responsive gene in breast cancer cells. Estrogen exposure resulted in ERRγ over-expression or translocation from the cytoplasm to the nucleus in breast cancer and EC, and ERRγ further mediates the cell proliferation promotion effects induced by estrogen (16,35). Apart from the crucial role of estrogen in cell growth, emerging evidence has indicated the involvement of estrogen in cell aggressiveness in certain types of cancers, such as breast, ovarian and EC, partially through cell stemness, motility and EMT promotion (36,37). In the present study, we found that ERRγ expression is stimulated dose- and time-dependently by estrogen in HEC-1A EC cells. In addition, ERRγ expression is unexpectedly correlated with S100A4 after different times of estrogen exposure. With consistent data from public datasets, we suspect that ERRγ may mediate estrogen signaling in EC progression by modulating S100A4 expression, which warrants further investigation.

In conclusion, for the first time, this study demonstrates that ERRγ is upregulated and positively related to S100A4 expression in EC. Additionally, ERRγ facilitates S100A4 transcription through promoter activation and promotes the migration and invasion capability of EC cell lines. Furthermore, the expression of both ERRγ and S100A4 could be regulated by estrogen stimulation. These findings extend our current knowledge of the mechanism of S100A4 regulation by transcription factors and suggest that ERRγ could be a potential novel therapeutic target in human EC.

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Availability of data and materials

The datasets used during the present study are available from the authors upon reasonable request.

Authors' contributions

HBW, YCZ and TH conceived and designed the study. TH, XXW, SQC and YL performed the experiments. DLF and YCZ were involved in data analysis. TH and XXW wrote the paper. HBW, YCZ and DLF reviewed and edited the manuscript. All authors read and approved the 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

The present study was performed in accordance with the Declaration of Helsinki, and approval to conduct the present study was obtained from the Ethics Committee of Tongji Medical College, Huazhong University of Science and Technology (IORG no: IORG0003571). Tissues were collected after receiving informed consent from the patients. All animal experiments were approved by the Animal Care Committee of Tongji Medical College.

Patient consent for publication

Not applicable.

Competing interests

The authors state that they have no competing interests.

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