Estrogen receptor $\beta$ upregulated by lncRNA-$H19$ to promote cancer stem-like properties in papillary thyroid carcinoma

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Abstract

Estrogen receptor $\beta$ (ER$\beta$) plays critical roles in thyroid cancer progression. However, its role in thyroid cancer stem cell maintenance remains elusive. Here, we report that ER$\beta$ is overexpressed in papillary thyroid cancer stem cells (PTCSCs), whereas ablation of ER$\beta$ decreases stemness-related factors expression, diminishes ALDH$^+$ cell populations, and suppresses sphere formation ability and tumor growth. Screening estrogen-responsive IncRNAs in PTC spheroid cells, we find that lncRNA-$H19$ is highly expressed in PTCSCs and PTC tissue specimens, which is correlated with poor overall survival. Mechanistically, estradiol (E2) significantly promotes $H19$ transcription via ER$\beta$ and elevates $H19$ expression. Silencing of $H19$ inhibits E2-induced sphere formation ability. Furthermore, $H19$ acting as a competitive endogenous RNA sequesters miRNA-3126-5p to reciprocally release ER$\beta$ expression. ER$\beta$ depletion reverses $H19$-induced stem-like properties upon E2 treatment. Appropriately, ER$\beta$ is upregulated in PTC tissue specimens. Notably, aspirin attenuates E2-induced cancer stem-like traits through decreasing both $H19$ and ER$\beta$ expression. Collectively, our findings reveal that ER$\beta$-$H19$ positive feedback loop has a compelling role in PTCSC maintenance under E2 treatment and provides a potential therapeutic targeting strategy for PTC.

Introduction

Papillary thyroid carcinoma (PTC) is one of the most common thyroid neoplasms, which exhibits multicentricity in the thyroid gland and frequently metastasizes to the regional lymph nodes, thereby increasing both morbidity and mortality. Increasing evidence indicates that papillary thyroid cancer stem cells (PTCSCs) play an important role in the progression of PTC. For example, stem cell marker $POUSF1$ is highly expressed in CD44$^+$/CD24$^-$ subpopulation and tumorigenic thyrospheroid cells from PTC. Tumor spheroids from PTC samples are more resistant to chemotherapeutics, including bortezomib, taxol, cisplatin, etoposide, doxorubicin, and vincristine, than non-spheroid PTC cells. In PTC tissues, a positive correlation has been found between stemness-related gene expression and tumor, lymph node, metastasis (TNM) staging. E2 is the most potent estrogen, which has a high affinity to estrogen receptor $\alpha$ (ER$\alpha$), estrogen receptor $\beta$ (ER$\beta$), and Peroxisome proliferator-activated receptor gamma (PPAR-$\gamma$ or PPARg). E2 enhances migration and invasion of PTC cells modulated...
by E-cadherin, vimentin and MMP-9. Moreover, E2 stimulation elevates stemness-related gene expression in PTC cells and promotes motility and tumorigenicity of PTSCCs in vivo. However, the molecular mechanism of estrogen regulating PTSCC maintenance remains poorly understood.

Long noncoding RNAs (lncRNAs) are a class of transcripts longer than 200 nucleotides but with no protein-coding potential, which play a crucial role in regulating cancer cell stemness. For example, recent studies show that knockdown of NEAT1 inhibits glioma stem cells progression via let-7e-NRAS axis. LncRNA H19 increases core pluripotency factor LIN28 expression by blocking the bioactivity of let-7 to promote breast cancer stem cell maintenance. LncRNA-DILC also attenuates liver cancer stem cell expansion through inhibiting the autocrine of IL6/STAT3 signaling. In addition, ElncRNA1 is transcriptionally regulated by E2 through ERα-estrogen response element pathway to promote epithelial ovarian cancer cell proliferation. Furthermore, E2 treatment also drives Sp1 to increase IncRNA MALAT1 expression and epigenetically controls various physiological processes of osteosarcoma cells. Although accumulating studies have indicated lncRNAs play important roles in maintaining CSCs and could be regulated by estrogen signaling in diverse cancers, little is known about the mechanism by which lncRNAs modulate E2-induced PTSCCs.

Emerged evidence has suggested that estrogen receptors (ERs) play pivotal roles in the pathogenesis of PTC. For example, ERα can trigger autophagy via activating ROS and ERK1/2 pathways to promote cell proliferation and inhibit apoptosis in PTC cells. ERβ is associated with apoptosis and growth inhibition, providing a negative correlation with mutant p53 in female PTC patients of reproductive age. Moreover, reciprocal interactions between ERβ and PPARg significantly inhibit PTC cell proliferation and migration, while ERα offsets the inhibitory effect of PPARg on cellular functions. In addition, ER-elevated OCT4 expression promotes self-renewal of the human breast cancer stem cells. Furthermore, thyroid stem and progenitor cells derived from nodular goiters express higher levels of ERα and ERβ compared with the differentiated thyrocytes. However, the underlying molecular mechanism whereby ER promotes PTC stemness is again still unclear.

Here, we demonstrate that ERβ is enriched in PTSCCs and contributes to PTSCC maintenance. Meanwhile, lncRNA H19 is highly expressed in PTSCCs and PTC tissue specimens. E2 promotes H19 transcription via ERβ. Ablation of H19 antagonizes E2-induced cancer stem-like properties in PTC cells. Moreover, ERβ is elevated through H19/miR-3126-5p signaling axis. ERβ depletion markedly reverses H19-mediated PTC stem-like capability under E2 treatment. ERβ is also upregulated in PTC tissue specimens. Importantly, aspirin suppresses E2-induced cancer stem-like characteristics through decreasing both ERβ and H19 expression. Taken together, our study identifies a novel mechanism of E2-induced ERβ-H19 positive regulatory circuit in PTSCC maintenance, providing a potential therapeutic strategy for PTC.

**Results**

**ERβ contributes to PTSCCs**

As the effect of estrogen is predominantly mediated through ERα and ERβ, we first examined whether ERα and ERβ are involved in PTC stemness. To this end, we performed sphere formation assay to enrich PTSCCs. The mRNA levels of ESR1 and ESR2 were compared between spheroid and monolayer cells. The results showed that ESR2 mRNA expression was remarkably elevated in both TPC-1 spheroid cells and K-1 spheroid cells compared to their monolayer counterparts (Fig. 1a). Spheroid cells exhibited much higher mRNA expression of stemness-related factors, including NANOG, SOX2, and POLIF51, in both TPC-1 and K-1 cells (Fig. 1b). Conversely, the spheroid cells exhibited a relative reduction of thyroid differentiation markers including Thyroglobulin (Tg) and thyroid stimulating receptor (TSHR) in both TPC-1 and K-1 cells (Supplementary Figure 1a). In addition, ERβ knockdown diminished ALDH+ cell populations in both K-1 cells (Fig. 1f) and TPC-1 cells (Supplementary Figure 1c). In addition, ERβ was knocked down by shRNA in both K-1 (Supplementary Figure 1d) and TPC-1 cells (Supplementary Figure 1e). Sphere formation assay showed that ERβ depletion significantly decreased spheroid numbers and diameters in both K-1 (Fig. 1g) and TPC-1 (Supplementary Figure 1f) cells. Moreover, the effect of ERβ on tumorigenesis was further examined in nude mice by injecting with control K-1 cells (NTC; non-targeting control), shERβ-1 K-1 cells (shERβ-1) and shERβ-2 K-1 cells (shERβ-2). As shown in Fig. 1h, the tumor volumes in shERβ-1 and shERβ-2 groups were apparently smaller than NTC group, indicating that ERβ is critical for PTSCC maintenance.

**H19 is highly expressed in PTSCCs and PTC tissue specimens**

The fact that lncRNAs can be induced by estrogen and are widely involved in cancer stem cell maintenance prompted us to investigate whether lncRNA plays a
Fig. 1 (See legend on next page)
critical role in regulating PTCSCs. To this end, we compared the expression levels of 13 potential estrogen-responsive lncRNAs (LCA1, H19, TC1500845, TC0101441, ROR, MALAT1, NEAT1, SRA1, HOTAIR, BC200, RP11-445H22.4, TC01000223 and TC01001686) between spheroids and monolayer cells.13,14,20–27 The results showed that H19 was the highest expressing lncRNA in spheroids as well as the highest differentially expressed lncRNA when compared with monolayer cells (Fig 2a, b). Consistently, H19 expression was much higher in spheroid cells than in monolayer cells through FISH assay (Fig 2c). Moreover, H19 expression level was significantly higher in PTC tissue specimens compared with the adjacent tissue specimens (Fig 2d, e). Furthermore, the Kaplan–Meier survival analysis demonstrated that high H19 levels were a strong indicator for poor overall survival of thyroid cancers in TCGA database (Fig 2f), suggesting a remarkably unfavorable prognosis and shorter lifespan. In summary, H19 is highly expressed in PTCSCs and PTC tissue specimens.

H19 depletion reverses E2-induced stem-like properties in PTC cells

To further explore whether H19 is involved in E2-induced PTSCC maintenance, we performed sphere formation assay upon treatment with E2 in PTC cells. Both sphere numbers and diameters were markedly elevated upon E2 treatment in both TPC-1 cells and K-1 cells (Supplementary Figure 2a and b). In addition, we conducted RT-qPCR assay to further explore the effects of E2 on PTC stemness. E2 increased substantially the mRNA expression levels of stemness-related factors, including NANOG, SOX2 and POLYSFI, in both TPC-1 cells and K-1 cells (Supplementary Figure 2c). PTC cells were also treated with various doses (0, 10, and 50 nM) of E2 for 36 h. As shown in Fig 3a, E2 significantly increased H19 RNA levels of PTC cells in a dose-dependent manner. Consistently, H19 RNA expression was also elevated by E2 treatment (50 nM) in a time-dependent manner in both TPC-1 cells and K-1 cells (Fig. 3b). Furthermore, E2 promoted H19 pre-RNA expression (Fig. 3c) and increased ESR2 but not ESR1 mRNA expression (Supplementary Figure 2d) in both TPC-1 and K-1 cells, which prompted that E2 regulates H19 transcription through ERβ. Indeed, silencing of ERβ significantly decreased both pre-H19 and H19 RNA levels (Fig. 3d). To determine transcription activity of H19, H19 promoter sequence (H19-WT) and the H19 promoter sequence with truncated ERE segment (H19-Del) and the H19 promoter with ERE domain (H19-Mut) were cloned into the pGL3 vector (Supplementary Figure 2e), respectively. E2 treatment promoted H19-WT luciferase activity, while it had no effects on H19-Del and H19-Mut activities (Supplementary Figure 2f and Fig. 3f). These data show that E2 promotes stem-like traits and increases H19 transcription in PTC cells.

We next investigated whether H19 mediated E2-derived cancer stem-like traits in PTC cells. To achieve this, H19 was knocked down by shRNA in TPC-1 cells and K-1 cells (Supplementary Figure 2g). Next, we performed sphere formation assay and observed that E2 treatment increased spheroid numbers and diameters, while H19 knockdown attenuated sphere formation capacity. Depletion of H19 significantly reversed E2-induced sphere formation capability in both K-1 cells (Fig. 3g) and TPC-1 cells (Supplementary Figure 2h). These data provide evidence to suggest that H19 plays an essential role in promoting cancer stem-like characteristics induced by E2 in PTC cells.

ERβ regulated by H19/miR3126-5p signaling axis promotes cancer stem-like properties upon E2 treatment

We next investigated the molecular mechanism whereby ERβ regulates H19-induced stem-like properties upon E2 treatment. We firstly measured the expression of ESR2 in the H19-knockdown (shH19) PTC cells. The result showed that there were no significant changes in ESR2 mRNA levels in the PTC cells upon H19 knockdown (Supplementary Figure 3a), while H19 depletion
**Fig. 2** *H19* expression is elevated in PTSCCs and PTC tissue specimens. 

**a** RT-qPCR analysis of the indicated lncRNA levels in the TPC-1 spheroid cells and TPC-1 monolayer cells. Data were shown as means ± SD (*n* = 3) *P* < 0.05, **P** < 0.01 and ***P*** < 0.001. 

**b** RT-qPCR analysis of the indicated lncRNA levels in the K-1 spheroid cells and K-1 monolayer cells. Data were shown as means ± SD (*n* = 3) *P* < 0.05, **P** < 0.01 and ***P*** < 0.001. 

**c** The in situ expression of *H19* RNA was detected by FISH assay. The red fluorescent represents *H19* RNA probe, and the blue fluorescent signal represents nuclear DNA counterstained with DAPI. The scale bar represents 10 μm. 

**d** *H19* expression in PTC tissues and adjacent normal tissues were analyzed by RT-qPCR assay (*n* = 38). The relative *H19* level was normalized to ACTB. The statistical difference was analyzed using the paired t-test. ***P*** < 0.001. 

**e** In situ analysis with a DIG-labeled *H19* probe in PTC tissue specimens and adjacent normal tissue specimens. The scale bar represents 100 μm. 

**f** Kaplan–Meier overall survival plots of 476 thyroid cancer patients created using PROGgeneV2, data set from TCGA-THCA. Patients were classified into *H19*-high and *H19*-low subgroups and analyzed as indicated.
Fig. 3 (See legend on next page.)
decreased ERβ protein levels in K-1 cells (Fig. 4a). E2 treatment increased ERβ expression, which could be attenuated by silencing H19 in K-1 cells (Fig. 4b and Supplementary Figure 3b). To confirm whether H19 acts as a competitive endogenous sponge interacts with miRNAs to release ERβ expression, we searched for miRNAs that interact with H19 and also target 3’UTR region of ESR2 by bioinformatic tools. The mimics of six identified miRNAs (Supplementary Figure 3c), including miR-4268, miR-3198, miR-876-3p, miR-1976, miR-3126-5p and miR-127-5p, were transfected into K-1 cells. The results showed that the miR-3126-5p mimic significantly decreased ERβ protein expression (Supplementary Figure 3d), while miR-3126-5p inhibitor remarkably increased ERβ protein level (Supplementary Figure 3e). Moreover, wild-type ESR2 3’UTR sequence including the putative miRNA-3126-5p response element (MRE) and the MRE mutant were cloned into the psiCHECK2 vector to give rise to psi-ESR2-WT and psi-ESR2-Mut (Supplementary Figure 3f), respectively. The psi-ESR2-WT and psi-ESR2-Mut vectors were then independently transfected into K-1 cells together with miR-3126-5p mimic or inhibitor in parallel with negative controls. The results showed that miR-3126-5p mimic repressed, but miR-3126-5p inhibitor increased, the relative luciferase activity of reporter psi-ESR2-WT, whereas both of them had no effects on psi-ESR2-Mut (Fig. 4c, d). Consistently, miR-3126-5p released by shH19 decreased ERβ expression, which could be rescued by the miR-3126-5p inhibitor in K-1 cells (Fig. 4e). Furthermore, we found that in K-1 cells the relative luciferase activity of psi-ESR2-WT (sensor) was induced by increasing amounts of wide-type H19 (H19-WT, sponge of miR-3126-5p), but not by H19 with the miR-3126-5p binding sites mutated (H19-Mut) in a dose-dependent manner (Fig. 4f).

Next, H19-overexpressing plasmid was transiently transfected into shERβ or non-targeting control (NTC) transduced K-1 cells (Supplementary Figure 3g). Sphere formation assay showed that E2 treatment or H19 over-expression significantly promoted sphere formation capacities, whereas depletion of ERβ restricted E2- or H19-induced stem-like properties in K-1 cells (Fig. 4g). These results support the idea that H19/miR3125-5p regulates stem-like properties upon E2 treatment through ERβ in PTC cells.

**ERβ is upregulated in PTC tissue specimens**

To further examine the ERβ expression in clinical samples, we performed immunohistochemistry (IHC) staining to measure ERβ in PTC tissue specimens and the corresponding adjacent tissues. ERβ exhibited higher expression in tumor tissue specimens compared to the corresponding adjacent tissues (Fig. 5a). Next, we assessed the expression of ERβ using western blotting assay in another six pairs of tumor tissue specimens, and similar ERβ expression patterns were also observed (Fig. 5b). These results demonstrate that ERβ is upregulated in PTC tissue specimens.

**Aspirin suppresses E2-induced cancer stemness through decreasing H19 and ERβ expression**

Previous studies have demonstrated that aspirin (ASA) possesses antineoplastic actions against a wide range of solid tumors. Upon ASA treatment, H19 expression was dramatically decreased in both dose-dependent (Fig. 6a) and time-dependent (Fig. 6b) manners. ASA also resulted in a decrease in the protein expression level of ERβ in a time-dependent manner (Fig. 6c). Moreover, H19-overexpressing plasmid in parallel with empty vector (EV) was transiently transfected into K-1 cells under ASA treatment. The expression of ERβ was rescued by over-expression of H19 in the presence of ASA (Fig. 6d and Supplementary Figure 4a). Notably, E2-enhanced sphere formation abilities were substantially attenuated by ASA in K-1 cells (Fig. 6e). In conclusion, these results reveal that H19 mediates E2-induced stem-like properties
Fig. 4 (See legend on next page.)
through upregulating ERβ expression in PTC cells, which can be inhibited by ASA (Fig. 6f).

**Discussion**

In this study, we demonstrate that the induction of ERβ expression by H19 is critical for PTCSC maintenance. In agreement, ERβ is highly expressed in PTCSCs and promotes PTC stem-like properties (Fig. 1). Screening estrogen-responsive lncRNAs in spheroid cells, we observe that H19 is significantly elevated in PTCSCs and PTC tissue specimens (Fig. 2). H19 transcription can be activated by ERβ under E2 treatment, and ablation of H19 reverses E2-induced stem-like traits in PTC cells (Fig. 3). Moreover, H19 sponges miR-3126-5p to release ERβ expression, and silencing of ERβ remarkably inhibits E2/H19-induced stem-like properties in PTC cells (Fig. 4). In concordance, ERβ also displays a higher expression level in PTC tissue specimens (Fig. 5). Notably, aspirin can antagonize E2-induced stem-like properties through suppressing H19 and thereby ERβ expression (Fig. 6).

Accumulating studies have revealed correlations between thyroid cancer incidence and ovulatory cycles, pregnancy, and lactation suppressant, which suggest a pivotal role of sex hormones in particular estrogen, in PTC progression. For example, E2 has been shown to stimulate thyroid cancer cell proliferation through increasing the anti-apoptotic protein BCL-2 and decreasing the pro-apoptotic BAX in an ERK1/2-dependent manner. E2 also promotes adhesion, migration, and invasion capacities via β-catenin in thyroid cancer cells. Recent studies have reported that estrogens are involved in elevating hematopoietic stem-cell self-renewal capabilities in female subjects and more specifically during pregnancy. Although E2 promotes sphere formation abilities, elevates tumorigenicity of PTCSCs and decreases the expression of the differentiation markers in thyroid progenitor cells. The detailed mechanisms in which estrogen modulates PTCSCs are still unknown. Recent study reported that H19 is downregulated in PTC tissues and PTC cell lines. In our study, only clinical specimens of reproductive age were selected as candidates, which were considered to have high estrogen levels. We found that H19 is upregulated in PTC tissues. In addition, H19 was elevated in PTCSCs enriched by sphere formation which indicates H19 plays an important role in PTC stemness. Conversely, silencing of H19 significantly reverses E2-induced PTC stem-like properties (Fig. 3). H19 has been reported to be upregulated by E2 via the estrogen–ERα–H19 signaling axis in breast tumors. Furthermore, CLIM interacting with ERα binds to H19 locus and promotes H19 expression, which negatively regulates corneal epithelial proliferation. However, the detail mechanism on how ER regulates H19 expression remains unclear. Our results show that E2 treatment increases IncRNA H19 transcription via ERβ in PTC cells. Thus, our data contribute to the understanding of the mechanism by which hormones effects on thyroid pathogenesis.

Recent studies have revealed that ERs play critical roles in the PTC development. ERα expression is usually increased in thyroid tumors, while ERβ expression is reduced when compared with normal parenchyma. Estrogen-activated ERα mediates the stimulatory effects on PTC proliferation and migration, whereas ERβ has inhibitory actions. In general, ERα promotes proliferation with an anti-apoptosis effect, while ERβ is related to apoptosis and growth inhibition. For this reason, the ERα/ERβ ratio is helpful in elucidating the thyroid cancer pathophysiology. However, ERβ expression was elevated in advanced prostate tumor tissues, which was associated with poor prognosis of hormone-naive patients. Depletion of ERβ attenuated mammosphere formation ability in breast cancer cells and patient-derived breast cancer cells. The fact that these two ERs have distinct distributions in the body and cell subpopulation indicates the different roles of ERα or ERβ maybe cancer-type and cell subpopulation-dependent. Our study firstly reveals that ERβ is highly expressed in PTCSCs and contributes to PTCSC maintenance (Fig. 1). ERβ depletion remarkably reverses H19-mediated PTC.
stem-like capability upon E2 treatment (Fig. 4). Our previous study has shown that H19 functions as a competitive endogenous RNA (ceRNA) to sponge miRNA let-7, leading to the upregulation of HIF-1α protein expression. Here, we demonstrate that E2-induced H19 acting as a ceRNA sponge miR-3126-5p to release ERβ expression. Furthermore, whether ERβ, as a key transcriptional factor, transactivates self-renewal genes to maintain PTCSCs requires further exploration.

As ERβ has a critical role in regulating PTCSC maintenance, targeting ERβ could provide a novel therapeutic avenue for advanced PTC patients. A specific ERβ antagonist, 4-[2-phenyl-5,7-bis(trifluoromethyl)pyrazolo[1,5-a]pyrimidin-3-yl]phenol (PHTPP), has been shown to be effective in many cancer types. For examples, bladder cancer burden and mortality can be controlled by PHTPP treatment in the carcinogen-induced bladder cancer models. Consistently, PHTPP can also attenuate 27-hydroxycholesterol-induced cell proliferation in prostate cancer cells. However, the clinical application of PHTPP has been limited by its high toxicities and inferior selectivity. In particular, a recent study has reported that
treatment with PHTPP even promotes prostate cancer invasion. As a result, new medications are urgently needed to replace PHTPP for targeting ERβ-induced CSCs. Accumulative evidence has demonstrated that the FDA-proved anti-inflammatory drug aspirin (ASA) can exert inhibitory effects on CSCs. For example, aspirin can...
restrict cancer stem-like properties by decreasing the expression of stemness-related factors in pancreatic cancer and has no significant toxic effects on normal cells. In addition, a previous study has also shown that aspirin inhibits breast cancer stem cell properties via targeting NF-kB signaling. Notably, our findings were the first to demonstrate that aspirin markedly inhibits PTC stemness through decreasing both lncRNA H19 and ERβ expression (Fig. 6).

In summary, our studies reveal that ERβ–H19 positive feedback loop promotes PTC stem-like traits under E2 treatment. In addition, this novel PTCSC regulatory mechanism could be inhibited by the clinically approved medicine aspirin, thus providing a potential therapeutic opportunity for aggressive PTC.

Materials and methods

Clinical samples

Following informed consent from patients and approved by the Institutional Ethics Review Board of first Affiliated Hospital of Dalian Medical University, all PTC samples and PTC paraffin tissue specimens used in this study were obtained from the first Affiliated Hospital of Dalian Medical University. Samples were frozen in liquid nitrogen immediately after surgical resection for later mRNA and protein extraction.

Cell lines

The human thyroid cancer cell lines (TPC-1 and K-1) and 293T cells were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cell lines were authenticated at ATCC before purchase by their standard short tandem repeat DNA typing methodology. Each cell line was cultured in its standard medium as recommended by ATCC. TPC-1 cells, K-1 cells, and 293T cells were maintained in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) FBS. All cells were incubated at 37 °C in a humidified incubator containing 5% CO2.

Chemicals and E2 treatment

All Chemicals including 17β-estradiol (E2), charcoal, and aspirin were obtained from Sigma (St. Louis, MO, USA). E2 was dissolved in ethanol and aspirin was dissolved in DMSO following manufacturer’s instructions. Before E2 treatment, cells were cultured in phenol red-free DMEM (Invitrogen, Carlsbad, CA, USA) medium supplemented with 10% charcoal-stripped FBS for three generations. Subsequently, we treated the cells with various concentrations of E2 as indicated and ethanol as vehicle control.

Sphere formation assay

Sphere formation assay was conducted in serum-free DMEM/F12 (Gibco, Carlsbad, CA, USA) supplemented with 2% (v/v) B27 (Invitrogen, Carlsbad, CA, USA), 20 ng/ml EGF (Sigma, St. Louis, MO, USA) and 20 ng/ml basic FGF (BD Biosciences, CA, USA). Dissociated single cells (600 or 1000) were seeded into 2 mL medium and propagated in six-well ultra-low attachment plates (Corning, NY, USA) and subsequently cultured at 37 °C in 5% CO2. Triplicate wells were set up. Sphere numbers were quantified at day 10 or 14. The spheres greater than 50 μm diameter were counted at 10× or 20× magnification under Olympus microscope.

RNA extraction and RT-qPCR analysis

Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer’s instructions, which was used to generate cDNA by using EasyScript One-Step gDNA Removal and cDNA Synthesis SuperMix Kit (TransGene Biotech, Beijing, China) with a random primer. RT-qPCR was performed using specific SYBR Select Master Mix (Invitrogen, Carlsbad, CA, USA) as recommended by the manufacturer. The relative mRNA levels were normalized to ACTB. The primers used were listed in Supplementary Table 1.

Western blotting

Cells were washed with ice-cold PBS and lysed in RIPA lysis buffer with freshly added cocktail protease inhibitor (Thermo Scientific, Rockford, IL, USA) on ice. Equal amounts of proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes (Millipore, County Cork, Ireland). The membranes were blocked with 5% fat-free milk in TBST at room temperature.
temperature for 60 min and then incubated with indicated primary antibodies followed by incubation with peroxidase-conjugated secondary antibodies (Thermo Scientific, Rockford, IL, USA) at room temperature for 60 min. The protein bands were detected and analyzed with an enhanced chemiluminescence kit (Amersham, Marlborough, MA, UK) using Bio-Rad ChemiDoc XRS+ Imaging System according to the manufacturer’s instructions. The Primary antibodies were used as follows: mouse anti-β-Actin (Proteintech, Wuhan, China), rabbit anti-ERβ (Bioworld Technology, Louis Park, MN, USA), rabbit anti-OCT4 (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-NANOG (Cell Signaling Technology, Danvers, MA, USA). 

**IHC staining and scoring**

For this trial, PTC paraffin tissues were sectioned into 5 μm slices. SPlink Detection Kits (Biotin-Streptavidin HRP Detection Systems, ZSGB-BIO, Beijing, China) and a DAB Kit (ZSGB-BIO, Beijing, China) were used. Briefly, xylene and gradient ethanol were used for dewaxing and dehydration respectively. To block endogenous peroxidase activity, slides were immersed in 3% hydrogen peroxide for 10 min. For epitope retrieval, slides were microwave treated in indicated target retrieval solution for 5 min, three times. After blocking, the slides were incubated with the primary antibody of ERβ (Abcam, Cambridge, MA, USA), NANOG (Abcam, Cambridge, MA, USA), overnight at 4°C, respectively. Then the slides were incubated with the HRP-labeled anti-rabbit IgG secondary antibody for 30 min and HRP for 20 min at room temperature respectively. Subsequently, DAB was used to stain the slides. Finally, the slides were counterstained with hematoxylin, dehydrated with gradient ethanol. Images were taken at 20× and 100× magnification by Olympus microscope. The immunostaining was observed and scored by two independent experienced pathologists using light microscopy (magnification 20×). The intensity of staining and the proportion of positive cells were used to evaluate the immunostaining. The staining intensity was graded as follows: absent staining as “0”, weak staining as “1”, intermediate staining as “2”, and strong staining as “3”. The percentage of positive cells score was ranked from 0 to 100%. Multiplying the percentage of positive cells score and the intensity score as the final score for each case. For ERβ, 67 was the median level of the final scores of all cases. For NANOG, 57 was the median level of the final scores of all cases. Stained tissues with a final score <median level was further classified as low, whereas tissues with a final score ≥median level were determined as high.

**ALDH staining**

For ALDH staining, the ALDH+ population was detected by ALDEFLUOR kit (Shanghai Stem Cell Technology Co. Ltd, Shanghai, China) following the manufacturer’s instructions. In brief, K-1 or TPC-1 (siNC and siERβ) cells (1 × 10^6/mL) were analyzed on a BD C6 flow cytometer (USA) after staining in ALDH1 substrate containing assay buffer for 30 min at 37°C in dark. The negative control was treated with diethylaminobenzaldehyde (DEAB), a specific ALDH inhibitor.

**Xenograft assay**

K-1 (NTC and shERβ) cells (1 × 10^6) were subcutaneously injected into BALB/c nude female mice (4–6 weeks old, n = 5). The tumor volumes were measured by calipers once every three days, estimated using the formula = 0.5 × a × b^2 (a and b were the long and short diameter of the tumors respectively). After 23 days, the mice were sacrificed, and the tumor xenografts immediately dissected.

**Fluorescent in situ hybridization**

A fragment of H19 designed as its probe was used and labeled with digoxigenin (DIG)-UTP (Roche, Mannheim, Germany) using the mMESSAGE T7 Ultra In Vitro Transcription kit (Ambion, Austin, TX, USA) following the manufacturer’s instructions. Slides were hybridized with probes overnight, washed twice with 50% formamide/2 × saline sodium citrate (SSC) and twice with 2 × SSC at 50°C for 5 min each time, then incubated with 1:500 diluted sheep anti-Dig (Invitrogen, Carlsbad, CA, USA) for 1 h at room temperature, followed by counterstained with DAPI (1 μg/ml), visualized using a confocal microscope (Leica, Wetzlar, Germany). Probe sequences were listed in Supplementary Table 1.

**Lentivirus infection and transient transfection**

Lentiviral-mediated short hairpin RNA (shRNA) directed against H19 and ERβ were purchased from Gene-Pharma, Suzhou, China. For shRNA lentiviruses infection, cells were infected in 6-cm dishes and subsequently split into 10-cm dishes in the presence of 2 μg/ml puromycin (Sigma, St. Louis, MO, USA) for selection over 72 h. The cells stably expressing shH19 or shERβ were chosen, respectively. shRNA sequence used were listed in Supplementary Table 1. Transient transfection was performed by using Lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturers’ protocols.

**Plasmids**

Promoters of H19 (−788/+44), H19 (−502/+44) were amplified from 293T genomic DNA and inserted into pGL3-Basic (Clontech, CA, USA) to generate the pGL3-H19-WT (−788/+44) and pGL3-H19-Del (−502/+44), respectively. The E2 responsive element (ERE) was mutated (pGL3-H19-Mut) by site-directed mutagenesis using PCR. Sequence of ESR2 3’UTR was amplified from 293T cDNA
and inserted into psiCHECK2 vector to generate psi-ESR2-WT. The miRNA response element (MRE) of miR-3126-5p in ESR2 3’UTR region was mutated (psi-ESR2-Mut) by site-directed mutagenesis using PCR. H19-expressing plasmids were constructed as previously described47. The miR-3126-5p binding sequence of H19 mutation (H19-Mut) was generated by site-directed mutagenesis using PCR. pRL-SV40 was purchased from (Clontech, CA, USA). All the primers used in plasmid construction were listed in Supplementary Table 1.

siRNAs, microRNA mimics, and microRNA inhibitors

siRNAs specifically targeting ERβ, siRNA control, miR-127-5p, miR-876-3p, miR-1976, miR-3126-5p, miR-3198, miR-4268 mimics and negative control, miR-3126-5p inhibitor and negative control were all purchased from GenePharma, Suzhou, China. All sequences were listed in Supplementary Table 1.

Dual-luciferase reporter assays

Luciferase activity was measured using the Dual-Luciferase Reporter Assay system (Promega, Madison, WI, USA) according to the manufacturer’s instructions. Growth media were removed, and cells were washed with cold PBS. Passive lysis buffer (200 μL per well) was added with gentle rocking for 15 min at room temperature. Lysates (50 μL) were transferred in black 96-well plate (Corning, NY, USA). Firefly and Renilla luciferase activity were assayed sequentially to the cell lysate in each well. For each luminescence reading, there would be a 2 s pre-measurement delay after injector dispensing assay reagents into each well, followed by a 10 s measurement time. For pGL3 reporter system, transcriptional activity was calculated as the ratio of firefly luciferase activity (reporter) to Renilla luciferase activity (control). For psiCHECK2 reporter system, the RNA stability was calculated as the ratio of Renilla luciferase activity (reporter) to firefly luciferase activity (control). Results represented the average of triplicate samples from three independent experiments.

Statistical analysis

Data were expressed as means ± SD of three independent experiments with GraphPad Prism software. The Student’s t-test was used to make a statistical comparison between groups. Pearson’s correlation test was used to examine the correlation between ERβ and NANOG by IHC staining. Statistical Package for Social Sciences (SPSS) software (version 24.0) was used for Statistical analysis in this study. *P < 0.05, **P < 0.01 and ***P < 0.001 were considered statistically significant.

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Conflict of interest

The authors declare that they have no conflict of interest.

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