

The role of 17 β -estradiol-induced upregulation of Piwi-like 4 in modulating gene expression and motility in breast cancer cells

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Abstract. A majority of breast cancer cases are positive for the estrogen receptor (ER), which means that they can respond to the estrogen hormone to achieve growth. Hence, the ER signaling pathway has been extensively targeted in pharmaceutical research and development in order to suppress tumor growth. However, prevalent hormone therapy and targeted therapy often become ineffective as cancer cells ultimately develop resistance, suggesting that there could be unidentified signaling molecules and events that regulate breast cancer growth. Notably, recent studies have uncovered that Piwi-like (Piwi) proteins, which were initially found in germline cells, are expressed in a wide spectrum of human cancers, including breast cancers. Although Piwi proteins have been well established to silence retrotransposons and to promote heterochromatin formation in germline cells, their somatic functions in cancer cells remain largely unknown. In the present study, we profiled the expression of four Piwi homologs in an ER-positive breast cancer cell line, MCF-7, and found that only Piwil4 was upregulated by 17 β -estradiol treatment. Notably, Piwil4 upregulation was not observed in an ER-positive but non-tumorigenic breast cancer cell line, MCF-12A. In addition, the induced expression of Piwil4 was dependent on estrogen/ER α signaling. To explore the biological significance of Piwil4 in breast cancer growth, we knocked down Piwil4 with multiple siRNAs and observed the suppressed expression of some canonical targets of ER. The knockdown of Piwil4 expression also decreased the migration and invasion capabilities of MCF-7 cells. Furthermore, the loss-of-function of Piwil4 reduced the motility of MCF-7 cells in wound-healing assays, which could be associated to decreased expression of vimentin and N-cadherin. Collectively, these findings revealed

that Piwil4 is a novel regulator of ER signaling that could be targeted to inhibit breast cancer growth and migration.

Introduction

Breast cancer has become a worldwide health problem for females. It is also a major cause of cancer-related deaths in women globally (1). Current treatment options include surgery, radiotherapy, chemotherapy, hormonal therapy and targeted therapy, the selection of which depends heavily on the classification of breast cancers (2-5). A commonly used criterion is the receptor status, namely whether the breast cancer is hormone receptor (estrogen and/or progesterone)-positive or hormone receptor-negative. For example, patients with estrogen receptor α (ER α)-positive tumors can be given tamoxifen (TAM) which is designed to antagonize the function of ER (6,7). They can also be treated with aromatase inhibitors, such as letrozole, which inhibits the synthesis of estrogen hormone. These patients have a better survival rate than those with ER α -negative tumors (8). In addition, targeted therapies, a form of precision medicine, such as palbociclib and ribociclib, have also been developed to treat those ER-dependent breast cancers. Although these therapies can suppress cancer growth for a significant period of time, very often they will be confronted with drug resistance acquired by cancer cells, indicating that there could be unknown signaling molecules and events in the ER cascade which warrant further exploration and characterization.

Recently, the Piwi (P-element-induced wimpy testis)-like proteins have gained much interest as prospective biomarkers and targets for anticancer therapies due to their ectopic expression in many types of cancers (9). Characterized by the presence of PAZ and PIWI domains, Piwi proteins belong to a subclade of the Argonaute family which also includes the AGO subclade (10). While AGO proteins are ubiquitously found in all tissues, Piwi proteins were initially regarded to be restricted to germline cells (11). The first Piwi gene in *Drosophila* was identified by screening for mutants affecting the asymmetric division of stem cells (12). Subsequently, identification of the Piwi homologs in a number of organisms has revealed that Piwi is evolutionarily conserved (13). In humans, there are four Piwi-like genes, namely, Piwi-like 1 (Hiwi, Piwil1), Piwi-like 2 (Hili, Piwil2), Piwi-like 3 (Piwil3)

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and Piwi-like 4 (Hiwi2, Piwil4) (14). In germ stem cells, Piwil proteins are involved in self-renewal, retrotransposon silencing, translational regulation and chromatin remodeling (15). Given that cancer cells share many characteristics with germ stem cells such as rapid proliferation and almost infinite self-renewal (11), it is therefore conceivable that Piwil proteins could be expressed in cancer cells and that some of the functions identified in germ stem cells could be hijacked by cancer cells for their own survival and metastasis.

Indeed, recent studies have detected the expression of Piwil proteins in a variety of somatic contexts, including cancers. For example, the expression of Piwil1 was observed in seminoma, a cancer of male germ cells (16). Furthermore, other members of the Piwi subclade have been found in many malignant tumors, including gastric, colon and breast cancers (9,17-21), and usually their expression is associated with poorer prognosis. A plethora of mechanisms have been proposed to explain the correlation, including epigenetic and post-transcriptional regulation (22-25). Piwil proteins can even either physically or functionally interact with some canonical signaling molecules and transcription factors, such as p38 and STAT3 (20,26).

Notably, a microarray profiling of breast cancer tissues indicated that Piwil3 and Piwil4 could serve as potential prognostic markers for breast cancer (27). Another study also revealed that Piwil4 was abundantly expressed in many breast cancer cases, particularly when only the triple-negative breast cancer (TNBC) samples were considered (21). By using a typical TNBC line, MDA-MB-231, the study revealed that Piwil4 was involved in regulating tumor invasion and growth via the TGF β and FGF pathways and in facilitating immune escape of cancer cells by suppressing the expression of MHC II. Hence, it is of great interest to further investigate whether in ER-positive cases, which represent a majority of the breast cancer population, Piwil4 or any other Piwi homologs play a functional role in modulating the ER signaling events.

In the present study, we found that in ER-positive MCF-7 breast cancer cells, 17 β -estradiol increased the expression of Piwil4, which was not observed in another ER-positive but non-tumorigenic breast epithelial cell line, MCF-12A. Conversely, the expression of Piwil4 was not induced by the antiestrogen TAM, but did depend on the presence of ER, particularly ER α . We further explored the effect of knocking down Piwil4 on ER transcriptional activity, and found that it led to reduced expression of some ER target genes. In functional assays, we observed that the knockdown of Piwil4 in MCF-7 reduced 17 β -estradiol-enhanced cell migration and invasion. The knockdown also impaired the wound healing motility of the cells, indicating that Piwil4 plays essential roles in modulating the response of ER-positive breast cancer cells to 17 β -estradiol in terms of target gene activation and migration capacity. These findings indicated that Piwil proteins could play a somatic role in regulating ER signaling activities, thus serving as a potential target for treating ER-positive breast cancers.

Materials and methods

Cell culture. The ER-positive breast cancer cell line MCF-7 and the non-tumorigenic breast cell line MCF-12A were

obtained from The American Type Culture Collection (ATCC; Manassas, VA, USA). MCF-7 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS; both from GE Healthcare, Chicago, IL, USA). MCF-12A cells were cultured in DMEM/F12 medium (GE Healthcare) supplemented with an FBS cocktail of 20 ng/ml Human epidermal growth factor (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 ng/ml cholera toxin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany), 0.01 mg/ml bovine insulin (Invitrogen; Thermo Fisher Scientific, Inc.), 500 ng/ml hydrocortisone (Invitrogen; Thermo Fisher Scientific, Inc.) and 5% horse serum (Gibco; Thermo Fisher Scientific, Inc.). Both cell types were grown in 75-cm² flasks (Corning Inc., Corning, NY, USA) in a humidified incubator under standard conditions at 37°C and 5% CO₂.

To activate the ER signaling in these cells, when the cultures reached 70% confluency, the cells were first washed and incubated with their corresponding media containing 5% charcoal-treated FBS (Sigma-Aldrich; Merck KGaA) for two days. Subsequently, 10 nM 17 β -estradiol (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was added to the culture medium at indicated time-points. Alternatively, 1 μ M tamoxifen (Sigma-Aldrich; Merck KGaA) in dimethyl sulfoxide (DMSO) was added to the culture medium for 1 or 3 h.

siRNA transfection. A day prior to transfection, MCF-7 cells were seeded into 6-well plates at a density of 3.5 x 10⁵ cells/well. Transfection of the cells with the siRNAs against Piwil4 (Sigma-Aldrich; Merck KGaA) and the siRNA Universal Negative (Qiagen GmbH, Hilden, Germany) was performed using Lipofectamine 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. In brief, for each well, 125 μ l Opti-MEM (Invitrogen; Thermo Fisher Scientific, Inc.) containing 5 μ l Lipofectamine 3000 was mixed with 125 μ l Opti-MEM containing 100 pmol siRNA in a microcentrifuge tube. Following incubation at room temperature for 5 min, the total mixture was added to the cells in the 6-well plate. The product information about the siRNAs used was as follows: i) siPiwil4-1: GUUACAAAG UCCUCCGGA; ii) siPiwil4-2: GCUUGAAGCCCCGACC AUAU; iii) esiPiwil4: EHU029551; iv) esiESR1: EHU141651; (all from Sigma-Aldrich; Merck KGaA); and v) siGENOME Human ESR2 SMARTpool: GGGCUGAUGUGGCGCUCA A; GUGUGAAGCAAGAUCGCUA; CAAGAAGAUUCC CGGCUUU; CUUUAGUGGUCCAUCGCCA (Dharmacon, Lafayette, CO, USA).

RNA extraction and quantification. Total RNAs from MCF-7 cells and MCF-12A cells were extracted with the TRIzol reagent (Life Technologies; Thermo Fisher Scientific, Inc.) as per the manufacturer's instructions. In brief, in a 6-well plate, each well received 1 ml TRIzol and then, 200 μ l chloroform was added per ml of TRIzol reagent for phase separation. Following centrifugation at 12,000 x g for 15 min at 4°C, 500 μ l of the aqueous phase was transferred to a new tube and mixed with 500 μ l isopropanol for RNA precipitation. Following centrifugation, the RNA pellet was washed with 70% ethanol and left to air dry. The RNA pellet was dissolved in 20 μ l RNase-free water (Promega Corp., Madison, WI,

USA) and the RNA concentration was assessed using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc.). The RNA was stored at -20°C for further use.

Reverse transcription-polymerase chain reaction (RT-PCR) and quantitative polymerase chain reaction (qPCR). For the reverse transcription reaction using the GoScript RT system (Promega Corp.), 3 µg of total RNA was pretreated with 1 µl Turbo DNase I (Ambion; Thermo Fisher Scientific, Inc.) at 37°C for 30 min to eliminate residual genomic DNAs. Following inactivation of DNase I with 5 mM EDTA at 70°C for 10 min, total RNA was mixed with 1 µl oligo(dT) and incubated at 70°C for 5 min. Following cooling on ice for 5 min, the sample was mixed with 9 µl of a Master Mix which contained 4 µl 5 X Reverse Transcription (RT) buffer, 2.5 µl 25 mM MgCl₂, 1 µl dNTP, 0.5 µl RNase inhibitor and 1 µl RT enzyme. The reaction was performed using a Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA, USA) at 25°C for 5 min followed by 42°C for 60 min and inactivated at 70°C for 15 min. The synthesized cDNAs were stored at -20°C for further use.

For the amplification of target genes, PCR was employed with the use of GoTaq DNA polymerase (Promega Corp.). A mixture of reaction components was prepared on ice from the following: 2.5 µl 10 X Standard Taq Reaction Buffer, 0.5 µl 10 mM dNTPs, 0.5 µl 10 µM forward primer, 0.5 µl 10 µM reverse primer, 1 µl GoTaq DNA polymerase, 1 µl of template cDNA and diluted with nuclease-free water to 25 µl. The reaction was performed on the thermal cycler (Bio-Rad Laboratories, Inc.) using the following cycling conditions: 30 cycles at 95°C for 20 sec, 60°C for 20 sec, and 72°C for 30 sec. The PCR products were then separated and analyzed in a 2% TAE agarose gel.

qPCR was performed in 96-well reaction plates (Life Technologies; Thermo Fisher Scientific, Inc.) on a real-time thermal cycler (7900HT Fast Real-Time PCR System; Applied Biosystems; Thermo Fisher Scientific, Inc.). Each well received 0.5 µl of cDNA template, 5 µl of 2 X GoTaq qPCR Master Mix, 0.25 µl 10 µM forward primer and 0.25 µl 10 µM reverse primer. The following reaction conditions were used. An initial polymerase activation at 95°C for 2 min followed by 40 cycles of denaturation at 95°C for 3 sec, annealing and extension at 60°C for 30 sec and one cycle of dissociation. β-actin mRNA expression was assessed and served as a reference gene which other mRNAs were normalized to. The relative abundance of mRNA was determined by the ΔΔCq method (28). A two-way Student's t-test for each gene was performed, and significant difference analyses were calculated using 95% confidence levels. The primer sequences for genes tested are listed below in the format of 'forward + reverse': β-actin, ggcatgggtcagaaggatt + aggtgtggtgccagattttc; GAPDH, tgccatgggtggaatcatattgg + gaaggtgaaggtcggagtcacac; Piwil1, cactgatggaagccagaagactc + gatcgttatcctcacatcctctcc; Piwil2, gtatcagcagagaagtggacaagc + agtcccaagactgaggtgttcc; Piwil3, atatctcgtcctcagtggttg + tccactctcgcgtcttttagtg; Piwil4, aatgctcgtttgaactagagac + attttgggttagtccacattaaat; Greb1, gagtggacaatgaggaagaggaag + ctcgttggaatggagacaagg; Pgr, cttaatacaactaggcgagag + aagctcatccaagaatactg; Cxcl12, attcttcgaaagccatgttgc + ttctccaggtactcctgaatcc; Tff1, ccattgacaacaaggtgatctgc + acagcagcccttatttgcacac; Nanog,

cacttctgcagagaagagtgtcg + gtactgtgaggttcaggatgttgg; c-Myc, tcagagtctggatcaccttctg + ggttggtgctgatctgtctcag; Oct4, gtgaagctggagaaggagaagc + gtcgtttggctgaataccttcc; Sox2, ccctgcagtacaactccatgac + tggagtggggaggaagaggtaac; Calcr, ccagtacatgatggcctgcaac + agtacacggcctggaatagc; Ccnd1, gcatgttcgtggcctctaag + cgtgtttgcggatgatctgt; vimentin, ggctcgtcaccttctgtgaat + gagaaatcctgctctcctcgc; N-cadherin, acatcttcagcgacgatagtcc + ctaattccatggcagctctgttcc; Esr1, ggatacgaagagaccgaagagg + gccaggctgttcttcttagagc; Esr2, tccttggtgtgaagcaagatc + atccttcacacgaccagactcc.

Protein preparation and immunoblotting. The MCF-7 cells were lysed in RIPA Buffer (Thermo Fischer Scientific, Inc.) supplemented with cOmplete™ protease inhibitor cocktail (Roche Diagnostics, Basel, Switzerland) and phosphatase inhibitors (Thermo Fischer Scientific, Inc.). The protein concentration was determined using the Bradford protein assay (Bio-Rad Laboratories, Inc.). Protein denaturation was performed by the addition of β-mercaptoethanol and 4 X NuPAGE® LDS Sample Buffer followed by 95°C for 10 min. Subsequently, 30 µg of lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (4-20% SDS-PAGE) and transferred onto PVDF membranes (Bio-Rad Laboratories, Inc.). The membranes were incubated with primary antibodies overnight at 4°C, rinsed 3 times with TBS-Tween followed by incubation with horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG (H+L) secondary antibody (1:200; cat. no. A16072; Thermo Fisher Scientific, Inc.) or goat anti-rabbit IgG (H+L) secondary antibody (1:200; cat. no. 31460; Thermo Fisher Scientific, Inc.). The immunoreactive bands were chemiluminescently detected using CL-Xposure™ Film (Thermo Fisher Scientific, Inc.). The primary antibodies used in the present study were anti-Piwi4 (1:100; cat. no. ab21869; Abcam, Cambridge, UK) and anti-β actin (1:200; cat. no. sc47778; Santa Cruz Biotechnology, Inc.).

Immunofluorescence. MCF-7 cells were seeded onto glass coverslips in 5% charcoal stripped FBS DMEM. Following 24 h, ethanol and 17β-estradiol were administered to the control and treatment groups respectively for another 24 h. The cells were rinsed twice with cold PBS, fixed *in situ* using 4% paraformaldehyde and permeabilized with 0.1% Triton X-100 in PBS. Cells were then rinsed thrice with PBS and blocked with 1% BSA (GE Healthcare) for 30 min. This was followed by incubation with primary antibody Piwi4 (1:100; cat. no. ab21869; Abcam) in 0.2% BSA for 2 h, 3 PBS washes and incubation with Alexa488-conjugated anti-rabbit IgG (1:200; cat. no. A11008; Invitrogen; Thermo Fisher Scientific, Inc.) in 0.2% BSA for 1 h in the dark. Counterstaining was performed with Hoechst 33342 (1:20,000; Sigma-Aldrich; Merck KGaA) in PBS for 5 min and rinsed with PBS thrice and desalted. Coverslips were left to dry and mounted onto glass slides using Prolong Gold Antifade medium (Molecular Probes; Thermo Fisher Scientific, Inc.). Images were captured using the Nikon Eclipse TE2000-S inverted microscope at x400 (Nikon Corp., Tokyo, Japan).

Transwell™ migration assay. The migration capability of transfected MCF-7 cells was studied using 8-mm Transwell migration chambers with 8-µm pores (Corning Inc.). At 24 h

post-transfection, the cell medium was removed, then the cells were rinsed once with serum-free DMEM and maintained in DMEM supplemented with 5% charcoal stripped FBS (Sigma-Aldrich; Merck KGaA) for another 24 h before trypsinizing. Prior to the seeding of cells, the bottom surface of each migration chamber was coated with 20 μ g/ml fibronectin (Corning Inc.) for 1 h. Cells (5×10^5) were seeded into the upper chambers in 200 μ l of DMEM supplemented with 5% of charcoal stripped FBS. DMEM (600 μ l) with 50% FBS was added to the lower chambers. Ethanol and 17 β -estradiol (Santa Cruz Biotechnology) was added to the medium of the control and treatment groups, respectively. The cells were allowed to migrate for 24 h. Chambers were then rinsed in cold PBS. Cells were fixed with 100% methanol and stained with 0.2% crystal violet. Cotton buds were used to remove the cells in the upper chamber. Chambers were left to dry overnight before imaging the migratory cells using the Nikon SMZ1500 inverted microscope (Nikon Corp.).

Cell invasion assay. The BD BioCoat Matrigel Invasion Chambers (BD Biosciences, Franklin Lakes, NJ, USA) used for the assay were first thawed to room temperature. To hydrate the chambers, 20 μ l serum-free DMEM was added to the upper chamber while 600 μ l PBS containing 20 μ g/ml fibronectin was added to the lower chamber. Fibronectin was used as an additional chemoattractant for the less invasive cell lines. The chambers were then maintained at 37°C in 5% CO₂ for 2 h. Each upper chamber then received 5×10^4 transfected MCF-7 cells suspended in 200 μ l serum-free medium while 600 μ l DMEM with 50% FBS was added to the lower chamber. Ethanol or 10 nM 17 β -estradiol was added to the upper and lower chambers of mock-treated or treated samples, respectively. The cells were then incubated at 37°C in 5% CO₂ for 24 h before being fixed and stained as described in the aforementioned migration assay.

Wound healing assay. Twenty-four hours post transfection, 4×10^4 MCF-7 cells in a total volume of 70 μ l DMEM containing 5% charcoal stripped FBS were seeded into each side of a 2-well cell culture insert (SPL Life Sciences, Pocheon, Korea) attached onto the surface of a 12-well plate. The cell culture inserts were removed after 24 h leaving an area without cells analogous to a wound. The well was then rinsed with serum-free medium and 1 ml of 5% charcoal stripped medium with ethanol or 17 β -estradiol was added. Wound healing images were captured using the Nikon Eclipse TE2000-S inverted microscope (Nikon Corp.) at time-points of 0, 12 and 24 h. The Nikon TI O2 inverted microscope (Nikon Corp.) was used to create the wound healing video by compiling images captured every 15 min for a duration of 24 h.

Statistical analysis. For statistical analysis of multiple groups in a dataset, one-way ANOVA was first performed in Excel. When the null hypothesis was rejected, we then performed a post hoc Student's t-test with Bonferroni correction to further analyze the statistical significance between two groups in the dataset. For the data set comprised of 3 groups (3 comparisons to be made), the Bonferroni-adjusted P-value thresholds were 0.0167 and 0.0033 to denote the significance levels of 5 and 1%, respectively. For the dataset comprised of 4 groups

(6 comparisons to be made), the adjusted thresholds were 0.00833 and 0.00167 to denote the significance levels of 5 and 1%, respectively.

Results

Piwi-like 4 expression is upregulated by 17 β -estradiol in MCF-7 cells. To investigate whether Piwi homologs are involved in the ER signaling pathway, we first characterized the expression of Piwil-4 in MCF-7 cells, which represent an ER-positive and epithelial-like breast cancer cell line. The quantitative PCR (qPCR) measurement of Piwil-4 transcripts revealed that Piwil4 expression was significantly elevated following a 3-h treatment with 10 nM 17 β -estradiol, while other Piwi homologs were not induced by the treatment (Fig. 1A), indicating Piwil4 could be specifically upregulated by the activated ER. To verify that the dose and duration of 17 β -estradiol treatment applied above could indeed activate ER signaling in MCF-7 cells, we assessed the transcript levels of some canonical ER target genes, including Greb1, Pgr, Cxcl12 and Tff1 (29-33). As anticipated, 17 β -estradiol could significantly upregulate these well-established targets (Fig. 1B). In addition, it has been demonstrated that 17 β -estradiol could increase the expression of stemness-related genes in breast cancer cells (34-36). Consistently, our experiments confirmed that Nanog, c-Myc, Oct4 and Sox2 transcript levels were elevated in 17 β -estradiol-treated MCF-7 cells (Fig. 1C), indicating that ER signaling was potentially activated.

To investigate whether the 17 β -estradiol-induced upregulation of Piwil4 is related to the tumorigenic cellular context of MCF-7 cells, we examined another ER-positive yet non-tumorigenic breast epithelial cell line, MCF-12A (37). Notably, Piwil4 was not induced by 17 β -estradiol after a 3-h treatment. Indeed, even after a 24-h treatment, no upregulation of Piwil4 was observed, while Greb1 and Cxcl12 were significantly induced (Fig. 1D). This observation indicated that the 17 β -estradiol-upregulated Piwil4 may be restricted to tumorigenic breast cells, hence raising an intriguing question as to whether Piwil4 is involved in tumorigenic ER signaling events.

Piwil4 is elevated by 17 β -estradiol and enriched in the nucleus of MCF-7 cells. Given that the Piwil4 transcript level was upregulated by 17 β -estradiol in MCF-7 cells, we further assessed whether the protein level of Piwil4 was similarly increased. As anticipated, the western blot analysis revealed that Piwil4 could be detected by a specific antibody in 17 β -estradiol-treated cells (Fig. 2A). In addition, the immunofluorescence staining showed that in the absence of 17 β -estradiol, Piwil4 was only faintly labeled and homogeneously distributed in the nucleus and cytoplasm of MCF-7 cells, and that 17 β -estradiol could elevate the level of Piwil4. Notably, Piwil4 was mostly enriched in the nuclei of 17 β -estradiol-treated cells (Fig. 2B), indicating a nuclear role, possibly related to transcriptional regulation, of Piwil4 in the ER signaling pathway.

Piwil4 induction is dependent on estrogen/ER α signaling. To investigate whether estrogen binding to ER is required for Piwil4 expression induction, MCF-7 cells were treated with

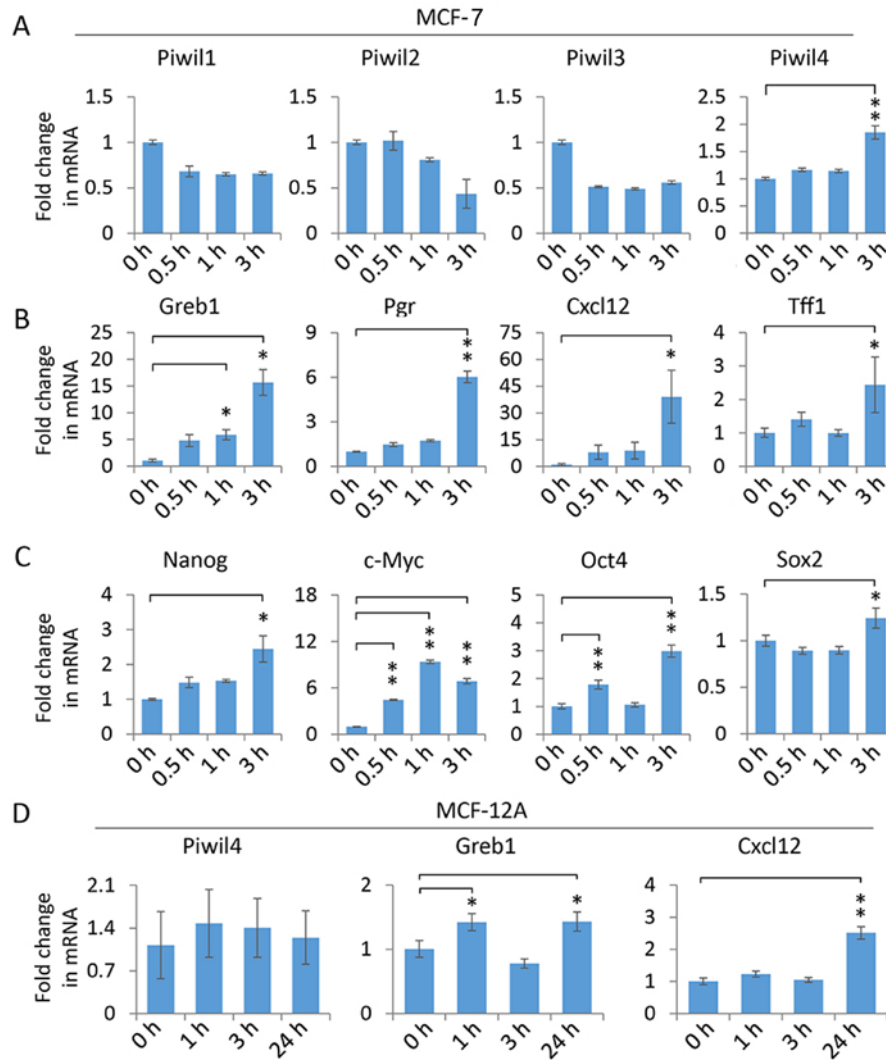


Figure 1. 17 β -estradiol upregulates the Piwil4 gene expression in MCF-7 cells. (A) MCF-7 cells treated with 10 nM 17 β -estradiol for indicated time-points were lysed for total RNA extraction. The transcript levels of Piwi homologs were determined by qPCR. β -actin was used as the reference gene for quantification. (B) The same RNA samples were analyzed by qPCR to quantify the transcript expression of ER target genes and (C) stemness-related genes. (D) The non-tumorigenic MCF-12A cells were treated with 10 nM 17 β -estradiol for 1, 3 and 24 h and the extracted total RNAs were analyzed by qPCR for Piwil4, Greb1 and Cxcl12. n=4; *P<0.00833, **P<0.00167 by Student's t-test with Bonferroni correction.

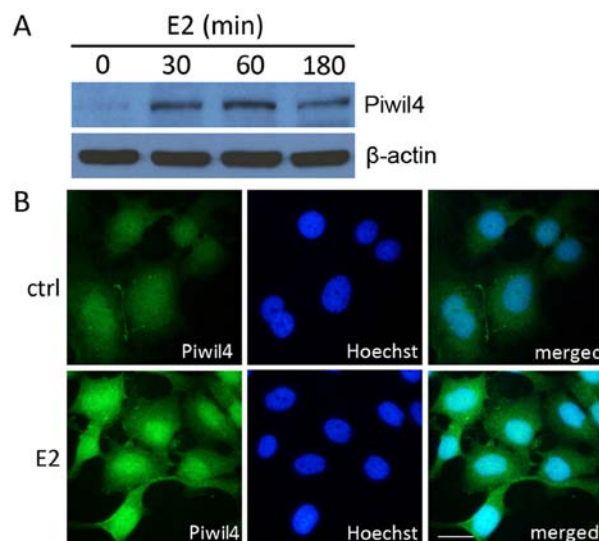


Figure 2. 17 β -estradiol increases the Piwil4 level in MCF-7 cells. (A) MCF-7 cells were treated with 10 nM 17 β -estradiol for indicated time-points and lysed in RIPA buffer. The protein lysate was analyzed by immunoblotting for Piwil4. β -actin was used as the loading control. (B) MCF-7 cells growing on coverslips were treated with 10 nM 17 β -estradiol and immunostained for Piwil4. The nucleus was counterstained with Hoechst 33342. Scale bar, 20 μ m. E2, 17 β -estradiol.

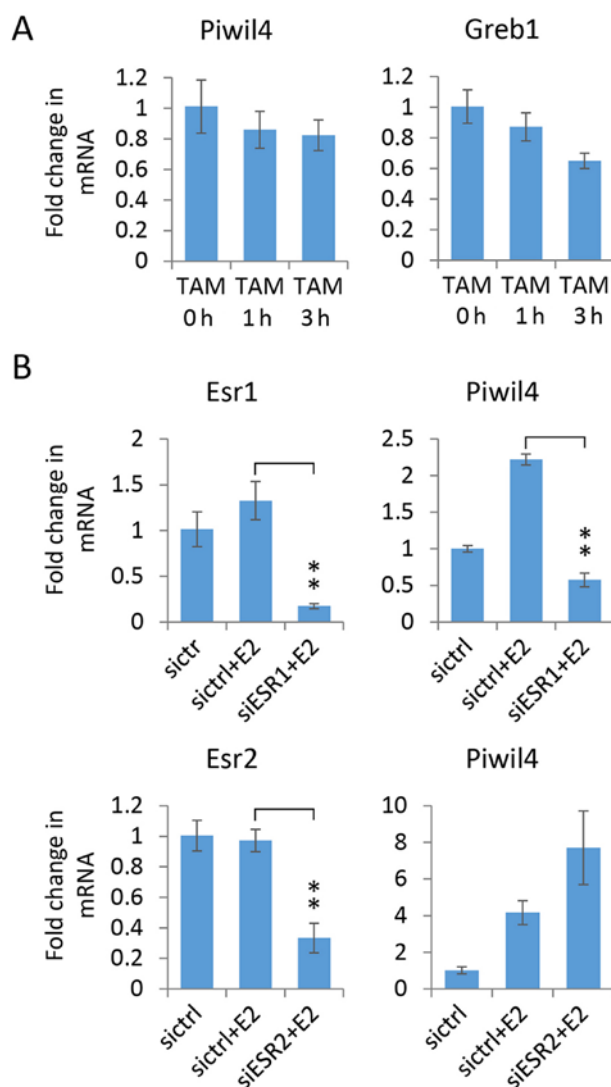


Figure 3. Piwil4 upregulation is dependent on estrogen/ER α signaling. (A) MCF-7 cells were treated with 1 μ M TAM for 1 or 3 h. The cells were then lysed for total RNA extraction. The transcript levels of Piwil4 and Greb1 were determined by qPCR. (B) MCF-7 cells were transfected with control siRNA (sictrl), siRNA pool targeting ER α (siESR1) or targeting ER β (siESR2), and then treated with 10 nM 17 β -estradiol for 3 h. The knockdown efficiency and Piwil4 transcript level were determined by qPCR. n=4; **P<0.0033 by Student's t-test with Bonferroni's correction. E2, 17 β -estradiol; TAM, tamoxifen.

TAM, a selective estrogen receptor modulator (SERM) (6,7). However, qPCR demonstrated that the expression of Piwil4, as well as Greb1, was not elevated by the treatment (Fig. 3A), indicating that binding of ER alone is not sufficient to increase the Piwil4 transcript level; the event is ligand-specific.

Of the two ERs, ER α (*Esr1*) and ER β (*Esr2*), MCF-7 cells mainly express the former (38). Hence, to elucidate whether ER, particularly ER α , is essential for estrogen-induced Piwil4 expression, we used specific siRNA pools to knock down ER α and ER β in MCF-7 cells which were then treated with 17 β -estradiol. Markedly, we found that 17 β -estradiol-induced Piwil4 upregulation was abolished in ER α -depleted cells, but not in ER β -depleted cells (Fig. 3B). The expression of Piwil4 was even higher in ER β -depleted cells, supporting the notion that ER β may antagonize ER α as previously suggested (38,39). In summary, these findings indicated that estrogen/ER α signaling is required for Piwil4 upregulation in MCF-7 cells.

Piwil4 knockdown affects the transcription of ER target genes in MCF-7 cells. To investigate the role of Piwil4 in regulating

ER signaling in MCF-7 cells, we performed the loss-of-function assays by using two gene-specific siRNAs, siPL4-1 and siPL4-2. The qPCR verification revealed that siPL4-1 could efficiently suppress Piwil4 expression at both the 1- and 3-h time-points following 17 β -estradiol treatment, while siPL4-2 demonstrated a very modest knockdown effect only at the 1-h time-point (Fig. 4A). Subsequently, when we assessed the transcript levels of some ER target genes following 3-h 17 β -estradiol treatment, it was noted that Greb1 (33), Tff1 (40), Calcr (31,41) and Ccnd1 (42,43) were significantly induced in MCF-7 cells. Notably, their upregulation was largely abolished by the suppression of Piwil4 using siPL4-1, but not siPL4-2 except for Greb1 (Fig. 4B), indicating that the transcriptional activation of some ER target genes was dependent on the expression of Piwil4.

To rule out the possibility of an off-target effect of siPL4-1, we used the siRNA pool (esiPL4) consisting of four different siRNAs against Piwil4. The reverse transcription PCR (RT-PCR) revealed that 17 β -estradiol could significantly increase the transcript level of Piwil4 and the induction

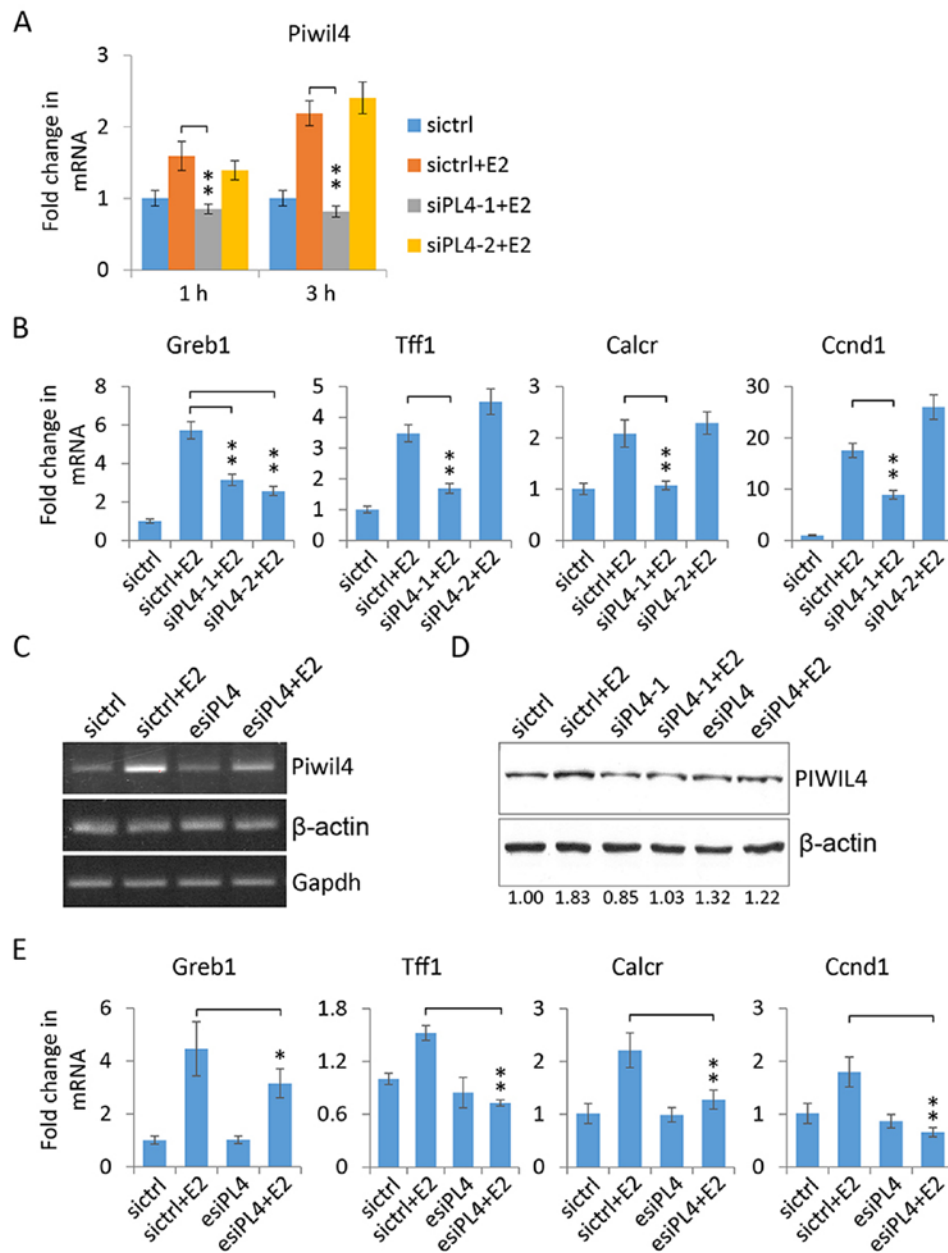


Figure 4. Piwil4 is needed for the expression of ER target genes. (A) Determination of the knockdown efficiency of two siRNAs targeting Piwil4 in MCF-7 cells. (B) The cells transfected with the two siRNAs were stimulated with 10 nM 17 β -estradiol for 3 h. The expression of some ER target genes was assessed by qPCR. (C) Verification of the knockdown efficiency of an siRNA pool targeting Piwil4 (esiPL4) by RT-PCR. β -actin and GAPDH were used as reference genes. (D) Immunoblotting confirmed the knockdown efficiency of siPL4-1 and esiPL4. The relative abundance of Piwil4 to β -actin was quantified by densitometry and the fold change was indicated below the β -actin strip. (E) Transfection of MCF-7 cells with esiPL4 downregulated the ER target genes, Greb1, Tff1, Calcr and Ccnd1. n=4; *P<0.00833, **P<0.00167 by Student's t-test with Bonferroni's correction. E2, 17 β -estradiol.

was largely abolished by esiPL4 (Fig. 4C). Immunoblotting confirmed that esiPL4, as well as siPL4-1, could suppress the induced expression of Piwil4 at the protein level (Fig. 4D). Consistently, esiPL4 transfection significantly reduced the 17 β -estradiol-induced expression of Greb1, Tff1, Calcr and Ccnd1 in MCF-7 cells (Fig. 4E), confirming the potential involvement of Piwil4 in regulating ER target gene expression.

Piwil4 knockdown decreases the migration and invasion of MCF-7 cells. We further employed esiPL4 to assess the role of Piwil4 in the regulation of the migration capability of MCF-7 cells. The transfected cells were seeded into the 8- μ m Transwell inserts. Similar to previous studies on the effect of estrogen on

breast cancer cell migration (44-46), 17 β -estradiol enhanced the migration of control siRNA (sictrl)-transfected MCF-7 cells across the substrate (Fig. 5A, upper panels). Notably, when Piwil4 was knocked down in MCF-7 cells by the transfection of esiPL4, 17 β -estradiol could no longer promote cell migration (Fig. 5A, lower left panel). The counting of migratory cells on the lower surface of Transwell inserts confirmed this observation (Fig. 5A, lower right panel), indicating that Piwil4 could be involved in 17 β -estradiol-mediated MCF-7 migration.

We then investigated whether Piwil4 could regulate the invasion behavior of MCF-7 cells by using the Matrigel-coated chamber. As MCF-7 is not a highly invasive breast cancer

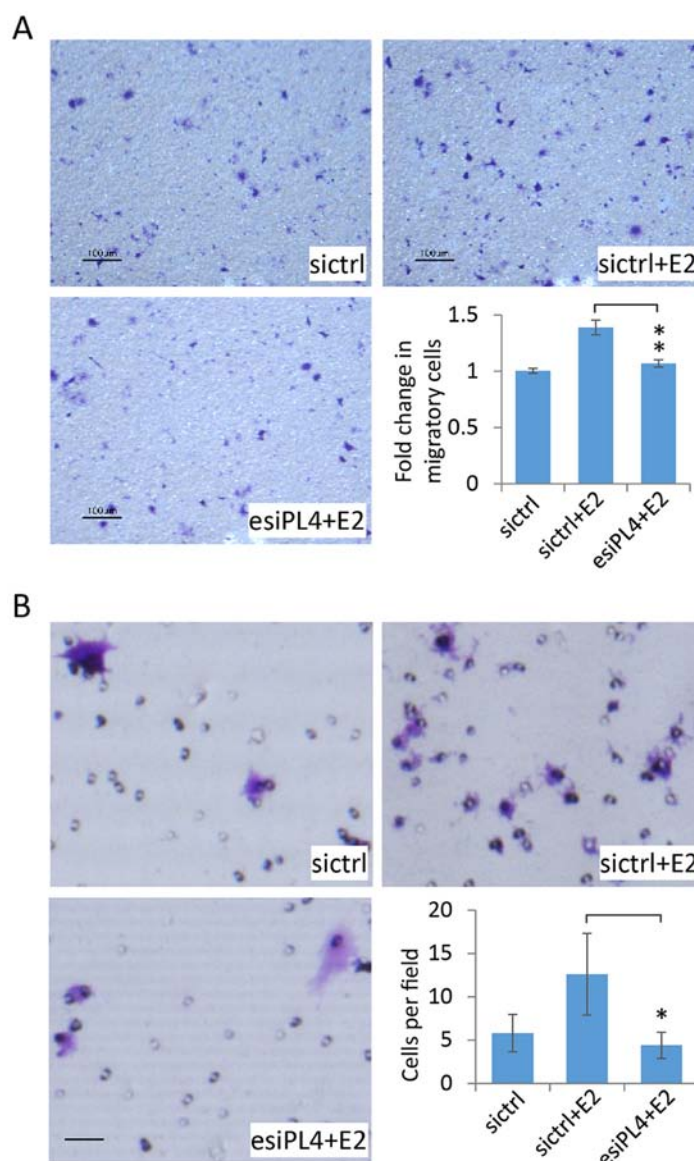


Figure 5. Piwil4 is involved in regulating migration and invasion of MCF-7 cells. (A) The esiPL4-transfected MCF-7 cells were seeded into 8- μ m Transwell inserts. The cells were allowed to migrate for 24 h with or without 17 β -estradiol. The migratory cells were stained with crystal violet. For each experimental condition, the cells from five random fields were counted. Scale bar, 100 μ m. (B) The esiPL4-transfected cells were seeded into Matrigel-coated inserts and incubated for 24 h in the absence or presence of 17 β -estradiol. The cells were then fixed and stained as described in the aforementioned migration assay. Cells from five random fields were counted. Scale bar, 20 μ m. n=5; *P<0.0167, **P<0.0033 by Student's t-test with Bonferroni's correction. E2, 17 β -estradiol.

cell line, the lower surface of the chamber was additionally coated with fibronectin which has been shown to promote the invasion of MCF-7 cells (47). We then observed that estradiol treatment could enhance the cell invasion as previously reported (36) (Fig. 5B, upper panels). In agreement with the migration assay (Fig. 5A), Piwil4 knockdown significantly reduced the invasion of MCF-7 cells (Fig. 5B, lower panels). This was reminiscent of a previous study that reported an essential role of Piwil4 in regulating the invasion capability of a TNBC cell line, MDA-MB-231 (21).

Piwil4 knockdown decreases the motility of MCF-7 cells in wound healing assays. To further assess the role of Piwil4 in the motility of MCF-7 cells, we performed wound healing assays by using esiPL4. Consistent to previous studies (44,45,48), it was noted that estrogen could enhance the motility and wound healing process of breast cancer cells, as evidenced by the

almost complete filling of the gap by the 17 β -estradiol-treated MCF-7 cells in a time-dependent manner (Fig. 6A, first two columns). Markedly, when Piwil4 was suppressed in MCF-7 cells, 17 β -estradiol enhanced the motility of the cells less effectively (Fig. 6A, last column). The measurement demonstrated that the wound gap was significantly wider in Piwil4-knockdown cells than in control-transfected cells 12- and 24-h following the scratch (Fig. 6B), implying an essential role of Piwil4 in regulating the motility of MCF-7 cells.

As vimentin has been reported to promote motility in several breast cancer cell lines, including MCF-7 and MDA-MB-231 (49,50), we further evaluated whether the observed effect of Piwil4 depletion on MCF-7 motility (Fig. 6A) was associated to vimentin. The qPCR data revealed that 17 β -estradiol significantly increased the mRNA level of vimentin, and that the knockdown of Piwil4 abolished the upregulation of vimentin (Fig. 6C). We also

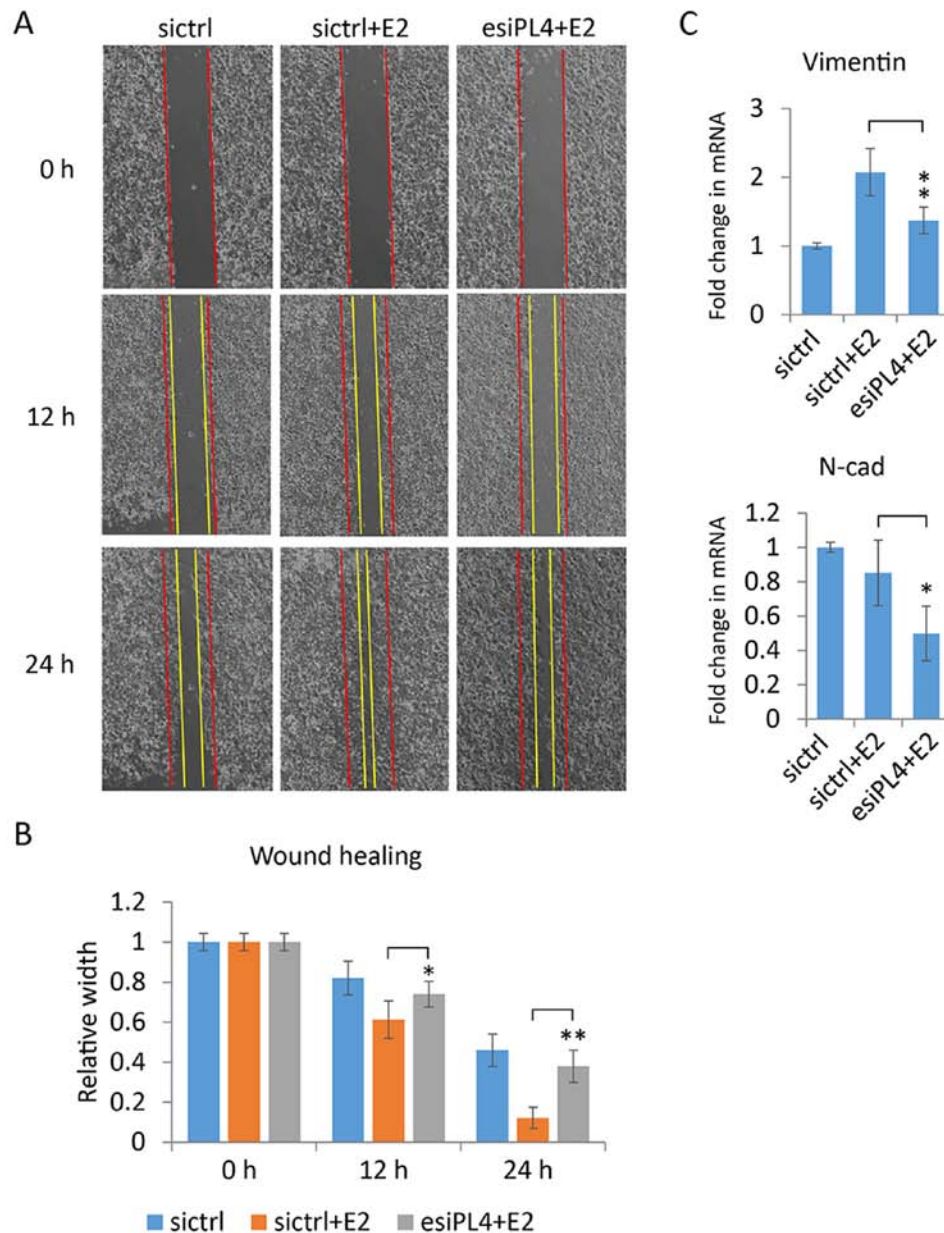


Figure 6. Piwil4 is involved in regulating the motility of MCF-7 cells. (A) The MCF-7 cells transfected with esiPL4 or control siRNA were subjected to wound-healing assays. The motility of MCF-7 cells was monitored and imaged every 15 min for a duration of 24 h. The red lines indicate the width of the original gap. The yellow lines denote the width of the diminishing gap. (B) The width of the gap was measured at 12 and 24 h following the scratch and normalized to the initial width. n=9; *P<0.0167, **P<0.0033 by Student's t-test with Bonferroni's correction. (C) The vimentin and N-cadherin (N-cad) transcripts were quantified in esiPL4-transfected MCF-7 cells by qPCR. n=4; *P<0.0167, **P<0.0033 by Student's t-test with Bonferroni's correction. E2, 17 β -estradiol.

assessed the transcript level of another mesenchymal marker, N-cadherin (21). Notably, the knockdown of Piwil4 reduced its transcript level in 17 β -estradiol-treated cells (Fig. 6C). These observations could explain the suppressed motility of Piwil4-depleted MCF-7 cells (Fig. 6A and B).

Discussion

In the present study, we examined the expression profile of Piwi homologs in an ER-positive breast cancer cell line, MCF-7, and we recorded an estrogen hormone-induced upregulation of Piwil4, but not of other Piwi homologs.

Typically, once activated by the estrogen hormone, ER will translocate into the nucleus, associate with a cohort of

coactivators, bind to the estrogen response element (ERE) and promote the transcription of target genes related to cell proliferation and growth (51). Indeed, the prediction with PROMO revealed that the proximal promoter region of *Piwil4* contains the potential binding sites of some classic transcription factors associated with ER, including AP-1 (52), C/EBP- β (53) and ETS1 (54). In addition, it is also conceivable that Piwil4 may be upregulated by an alternative, non-genomic ER signaling cascade. In that case, the hormone receptor could directly interact and activate mitogen-activated protein kinases (MAPK), phosphatidylinositol 3-OH kinase (PI3K) and tyrosine kinases (55-57), which could subsequently initiate downstream events to regulate cellular functions.

Notably, Piwil4 was not similarly upregulated by 17 β -estradiol in another ER-positive but non-tumorigenic breast cell line, MCF-12A cells, raising an interesting question whether the induced expression of Piwil4 is restricted to tumorigenic cells. In fact, a recent study revealed a globally higher level of Piwil4 in breast cancer samples than in normal breast tissues (21). Hence, it is conceivable that Piwil4 could play some essential role in tumorigenic signaling activities.

Indeed, in Piwil4-depleted MCF-7 cells, some ER target genes were significantly less induced, including Tff1, Greb1, Ccnd1 and Calcr (Fig. 4), indicating that Piwil4 was involved in modulating ER signaling. Similarly, a recent study identified a global alteration in gene expression in Piwil4-depleted MDA-MB-231 cells (21). A subsequent pathway analysis revealed that the most affected genes were enriched in the TGF β and FGF pathways. It would be interesting to further explore the distinct populations of Piwil4-regulated targets in different types of breast cancers so that the novel function of Piwil4 can be precisely targeted.

Furthermore, by knocking down Piwil4 in MCF-7 cells, our functional assays also revealed that Piwil4 was involved in regulating the migration and invasion of breast cancer cells, and that the regulation could be achieved via two mesenchymal markers, vimentin and N-cadherin (Figs. 5 and 6). This observation indicated that Piwil4 could be targeted to limit the migratory ability of cancer cells, and to even prevent the epithelial-mesenchymal transition. Indeed, in the TNBC cell line, MDA-MB-231, the depletion of Piwil4 was also reported to reduce the expression of N-cadherin and metastasis (21).

In summary, the present study revealed an estrogen-induced upregulation of Piwil4 in breast cancer cells. The loss-of-function assays further indicated that Piwil4 may be involved in regulating the expression of ER target genes and the migratory ability of breast cancer cells. Future studies are warranted to characterize the potential interaction between Piwil4 and canonical ER signaling components so that Piwil4 can be further exploited to treat breast cancer.

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Availability of data and materials

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

Authors' contributions

ZSLH and JYL performed the experiments. CSS, CW and LZT initiated the project and provided technical support for

immunoblotting and immunofluorescence. QH designed the experiments, analyzed the data and wrote 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

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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