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<tr>
<td>Citation</td>
<td>Oncotarget, 2015, v. 6 n. 25, p. 21704-21717</td>
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<tr>
<td>Issued Date</td>
<td>2015</td>
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<tr>
<td>URL</td>
<td><a href="http://hdl.handle.net/10722/217240">http://hdl.handle.net/10722/217240</a></td>
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ERα inhibits epithelial-mesenchymal transition by suppressing Bmi1 in breast cancer

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Keywords: ERα signaling, epithelial-mesenchymal transition, Bmi1, breast cancer, stemness

Received: October 20 2014   Accepted: April 30, 2015   Published: May 13, 2015

ABSTRACT

In human breast cancer, estrogen receptor-α (ERα) suppresses epithelial-mesenchymal transition (EMT) and stemness, two crucial parameters for tumor metastasis; however, the underlying mechanism by which ERα regulates these two processes remains largely unknown. Bmi1, the polycomb group protein B lymphoma Mo-MLV insertion region 1 homolog, regulates EMT transition, maintains the self-renewal capacity of stem cells, and is frequently overexpressed in human cancers. In the present study, ERα upregulated the expression of the epithelial marker, E-cadherin, in breast cancer cells through the transcriptional down-regulation of Bmi1. Furthermore, ERα overexpression suppressed the migration, invasion, and EMT of breast cancer cells. Notably, overexpression of ERα significantly decreased the CD44high/CD24low cell population and inhibited the capacity for mammosphere formation in ERα-negative breast cancer cells. In addition, overexpression of Bmi1 attenuated the ERα-mediated suppression of EMT and cell stemness. Immunohistochemistry revealed an inverse association of ERα and Bmi1 expression in human breast cancer tissue. Taken together, our findings suggest that ERα inhibits EMT and stemness through the downregulation of Bmi1.

INTRODUCTION

Of all cancers, that of the breast is the most common for women, with most breast cancer-related deaths involving widespread metastasis [1]. Estrogen receptor-α (ERα) is a nuclear receptor that is activated by the sex hormone, estrogen; it regulates the transcription of estrogen-responsive genes in diverse target cells. Ligand binding induces a conformational change within ERα, thus promoting dimerization and high-affinity binding to specific estrogen-responsive elements (EREs) located within the promoter of target genes [2]. ERα is an important prognostic indicator in breast cancer [3]. ERα signaling promotes the growth of primary breast cancers, but can also antagonize signaling pathways that lead to epithelial–mesenchymal transition (EMT) [1, 2, 4–7]. ERα-positive breast cancer cells appear to contain a relatively small subpopulation of breast cancer stem cells [8, 9]. Post-EMT breast cancer cells express cancer stem cell markers, including Bmi1, but show decreased ERα expression [10, 11]. However, the mechanisms by which ERα regulates EMT, as well as inhibits stemness in breast cancer, remain to be explored.

In EMT, epithelial cells lose their polarity and acquire the migratory and invasive properties of mesenchymal cells. Cell-to-cell adhesion is mediated by cadherins such as E-cadherin, which provides a structural support for cell–cell attachment. Thus, a loss
of E-cadherin expression can cause a loss of polarity of epithelial cells and is considered a fundamental event in EMT [12, 13]. In addition to its crucial role in the differentiation of many tissues and organs, EMT has also been shown to cause organ fibrosis and promote carcinoma progression through a variety of mechanisms [13]. Recent reports suggest a direct link between EMT and the gain of stem-like properties [12, 13]. Induction of EMT not only allows cancer cells to disseminate from the primary tumor, but also promotes their self-renewal capability. In breast cancer, EMT is associated with cancer stem cell properties, including the expression of a stem cell-associated CD44<sup>high</sup>/CD24<sup>low</sup> antigenic profile, and self-renewal capabilities [10]. Despite this, the molecular pathways linking EMT to the acquisition of stem cell properties remain, as yet, largely undefined.

Increasing evidence suggests that the polycomb group of transcription factor proteins plays a crucial role in cancer development and recurrence. Of these, Bmi1 is a member of the polycomb-repressive complex 1, which is strongly involved in the self-renewal of stem cells and is associated with a number of human malignancies such as oropharyngeal cancer, neuroblastoma and melanoma [14–18]. Bmi1 maintains the self-renewal of both normal and malignant human mammary stem cells [19], suppresses E-cadherin, and enhances stemness in head and neck cancer cells [20]. Bmi1 also plays an important role in both EMT and stemness processes in human nasopharyngeal and pancreatic cancers [21, 22]. In a previous study, ERα was demonstrated to up-regulate the expression of E-cadherin both directly and indirectly [2, 7, 23, 24]. However, whether EMT inhibition by ERα is mediated through a direct/indirect upregulation of E-cadherin remains poorly understood, and further, the regulatory mechanism of Bmi1 in cancer cells and its role in metastasis are largely undefined.

We hypothesized that ERα may upregulate E-cadherin through a Bmi1-mediated pathway. We investigated this process in vitro in breast cancer cells, and in vivo in a mouse model and human breast cancer tissues.

**RESULTS**

**Estrogen hormone (E2) and ERα downregulates Bmi1 expression and increases E-cadherin expression in breast cancer cells**

As was previously reported, post-EMT breast cancer cells express cancer stem cell markers, including Bmi1, but display decreased ERα expression [1]. In order to quantify Bmi1 expression in breast cancer cells, we detected Bmi1 protein expression by Western blot in various breast cancer cell lines. We found that Bmi1 expression was higher in three ERα-negative breast cancer cell lines (SKBR3, BT549, and MDA-MB-231) than in ERα-positive T47D or BT474 cells (Figure 1A). To compare Bmi1 mRNA expression in these lines, real-time RT-PCR was performed, with β-actin used as an internal control. Consistent with Bmi1 protein expression, Bmi1 mRNA levels were 2 to 3 fold higher in ERα-negative breast cancer cell lines than in in ERα-positive cells (Figure 1B).

Because both protein and mRNA levels of Bmi1 were decreased in ERα-positive T47D cells relative to ERα-negative breast cancer cell lines, we determined whether ERα signaling played a role in Bmi1 expression. T47D cells cultured in estrogen-depleted medium were treated with various concentrations of E2. After 24 or 72 h, Bmi1 protein and mRNA levels were dose-dependently downregulated by E2 (Supplemental Figures 1A, 1B; Figures 1C, 1D, respectively). When the cells were treated with E2 at 10<sup>−7</sup> M for 72 h, Bmi1 mRNA levels were significantly reduced by approximately 70% (Figure 1D; P < 0.05), and protein levels were decreased by more than 90% (Figure 1C).

To further investigate the impact of ERα on Bmi1, we silenced endogenous ERα in T47D cells using siRNA and examined Bmi1 and E-cadherin expression. As shown in Figures 1E and 1F, silencing endogenous ERα in T47D cells led to the significant up-regulation of Bmi1 in Figures 1G and 1H, respectively). We further analyzed the expression of Bmi1 and E-cadherin. Bmi1 was markedly downregulated and E-cadherin was upregulated at both the protein level, and significantly, at the mRNA level in pEGFP-C1-ERα, as compared with pEGFP-C1-transfected BT549 cells (Figures 1G, 1H; P < 0.05 for mRNA).

**ERα down-regulates Bmi1 expression by directly binding to the BMI1 promoter**

Based on our previous findings that Bmi1 expression is transcriptionally regulated by ERα signaling, we addressed whether ERα can directly bind to the regulatory regions of the BMI1 promoter. To determine the binding site, we searched for specific EREs located within the BMI1 promoter. We did not find classical ERE sites but instead found a half-ERE site at position −178 to −174 (Figure 2A). To investigate whether ERα could form a complex with the BMI1 promoter, we performed a chromatin immunoprecipitation (ChIP) assay with primers covering the BMI1 promoter region. We used a far upstream region, beyond the half-ERE site, in the BMI1 promoter as a negative control. ERα bound to the region between positions −237 to −106 containing the half-ERE...
site, but not the region between positions −1184 to −1023, which did not contain the ERE/half-ERE binding site (Figure 2B).

Electrophoretic mobility shift assay (EMSA) revealed an ERα-binding band after the incubation of nuclear extracts from BT549 cells overexpressing ERα.
Figure 2: Bmi1 expression is directly regulated by ERα. A. A schematic representation of the BMI1 promoter region, with or without the half-ERE site for ERα. Precipitated DNA was amplified by PCR with specific primers for regions 1–2. B. Chromatin immunoprecipitation (ChIP) assay involved normal IgG (IgG), or anti-ERα (ERα) antibody to identify the ERα binding site on the BMI1 promoter in T47D cells. C. Electrophoretic mobility shift assay (EMSA). Nuclear extracts from pEGFP-C1-ERα-transfected BT549 cells were incubated with a probe labeled at its 3′-end with biotin from the BMI1 promoter region containing the ERα binding site (lanes 1–8). Unlabeled probe (lane 1) or mutant oligonucleotides (lane 3) were added at 50-fold greater concentrations than that of the labeled probe. In the non-specific binding assay, 25 or 125 ng of poly (dI: dC) (lanes 4–5), normal IgG (lane 6), or anti-Twist1 antibody (lane 7) was added. For the supershift assay, the addition of anti-ERα antibody resulted in a supershifted band (lane 8). D. T47D cells were co-transfected with a BMI1 promoter construct (pXP2-BMI1–Luc1000) and ERα siRNA (3 or 15 nM). E. T47D cells were transfected with a BMI1 promoter construct (pXP2-BMI1–Luc1000) and treated with either ethanol vehicle (EtOH) or 100 nM E2 (E2) for 24 h. F. T47D cells were transfected with pXP2-BMI1–mutant and treated with either ethanol vehicle (EtOH) or 100 nM E2 (E2) for 24 h. G. BT549 cells were co-transfected with pXP2-BMI1–Luc1000 and either pEGFP-C1 or pEGFP-C1-ERα. H. BT549 cells were co-transfected with pXP2-BMI1–mutant and either pEGFP-C1 or pEGFP-C1-ERα. E–H, All cells were co-transfected with Renilla luciferase plasmid. Luciferase activity was normalized to that of Renilla. *P < 0.05 (Student’s t test) compared with control cells. Data are mean ± SEM (n = 3).
with labeled oligonucleotides containing the half-ERE from the BMI1 regulatory region; a supershifted band was seen after the addition of an anti-ERα-specific antibody to the mixture (Figure 2C). The addition of excess amounts of unlabeled oligonucleotides containing the half-ERE outcompeted and abolished the binding activity of ERα, but not the addition of mutant half-ERE–containing unlabeled oligonucleotides or non-specific competition by poly (dI:dC). No supershifted band was seen after the addition of an unrelated antibody, normal IgG, or anti-Twist1 antibody, which binds to the E box of the BMI1 promoter.

To determine the direct regulation of BMI1 by ERα, we used a luciferase reporter vector that included the region from −1000 to −1 of the BMI1 gene. A dual luciferase reporter assay was used to investigate BMI1 promoter activity in ERα-silenced T47D cells or ERα-overexpressing BT549 cells by co-transfection with the BMI1 promoter and Renilla luciferase reporter genes. Transfection with ERα siRNA significantly increased BMI1 promoter activity 2- to 3.5-fold in T47D cells in a dose-dependent manner (Figure 2D; *P* < 0.05). E2 administration decreased wild-type BMI1 promoter activity to 0.5-fold (Figure 2E; *P* < 0.05) but did not show any effect on the mutant BMI1 promoter (Figure 2F). Similarly, ectopic ERα overexpression significantly decreased BMI1 promoter activity to 0.5-fold in BT549 cells (Figure 2G; *P* < 0.05), but showed no effect on mutant BMI1 promoter activity (Figure 2H). These results demonstrated that ERα directly repressed BMI1 transcription by specifically binding to the promoter region of the BMI1 gene.

**ERα suppresses EMT and stemness in breast cancer cells**

We next examined BT549 cells, stably transfected with either pEGFP-C1 or pEGFP-C1-ERα vector, for morphological changes indicating EMT (Figure 3A). pEGFP-C1 BT549 cells possessed a fibroblast-like morphology and displayed pronounced cellular scattering, while pEGFP-C1-ERα BT549 cells expressing ERα showed a morphological conversion from a spindle-shaped, mesenchymal morphology to a cuboidal, epithelial morphology (Figure 3A). Through seeding small amounts of cells, apparent morphological change could be seen after 48 h culture (Supplemental Figure 2). In contrast, silencing endogenous ERα expression in T47D cells resulted in a partial loss of epithelial characteristics (Figure 3B).

To further investigate the impact of ERα on EMT, we determined migratory and invasive behaviors in ERα stably-transfected BT549 cells. The healing of wounded areas was significantly slower by about 65% in pEGFP-C1-ERα, compared to pEGFP-C1, BT549 cells (Figure 3C; *P* < 0.05). Overexpression of ERα in BT549 cells significantly decreased the level of migration to about 50% of the control (Figure 3D; *P* < 0.05). Matrigel invasion chamber assays revealed that overexpression of ERα markedly reduced, to about 35% of capacity, the invasiveness of BT549 cells (Figure 3E; *P* < 0.05).

Post-EMT mammary epithelial cells show an increased capability of mammosphere formation and an increased CD44high/CD24low phenotype, both of which are characteristics of normal mammary and breast cancer stem cells [14–16]. The mammosphere formation assay showed a significant decrease in both the size and number of mammospheres in pEGFP-C1-ERα BT549 cells (Figure 3F; *P* < 0.05 and Supplemental Figure 3). To determine whether ERα expression decreased the cancer stem cell–like cell population by suppressing EMT, we analyzed the CD44high/CD24low cell population in ERα stably-transfected BT549 cells. Most of the control pEGFP-C1 BT549 cells showed a CD44high/CD24low phenotype, which was significantly decreased, from 76% to 7% of the cell population in pEGFP-C1-ERα BT549 cells (Figure 3G; *P* < 0.05). These results indicate that ERα suppresses both the expression of cell surface markers and functional characteristics associated with cancer stem cells.

**ERα decreased the metastatic ability of BT549 cells in vivo**

To test whether ERα overexpression indeed decreases the metastatic ability of BT549 cells in vivo, a tail vein metastatic model was established in BALB/c nude mice. At 6 weeks following the injection of tumor cells, animals were sacrificed and lung metastasis was examined by H&E staining. Three of the five mice implanted with BT549 cells developed lung metastases. In contrast, mice implanted with BT549 cells overexpressing ERα showed no evident metastatic nodules in lungs (Figures 4A, 4B). Experimental and control groups did not show metastasis in the liver (data not shown).

**Restoration of Bmi1 expression reverses ERα-mediated suppression of EMT and stemness**

Next, we investigated the role of Bmi1 in ERα-mediated suppression of EMT and stemness properties in breast cancer cells. A Bmi1 expression vector or empty vector was transiently transfected into pEGFP-C1-ERα BT549 cells; restoration of Bmi1 expression resulted in the loss of E-cadherin expression (Figures 5A, 5B), significantly increased migration or invasion (Figures 5C, 5D; *P* < 0.05 for both), and significantly increased mammosphere-forming ability (Figure 5E; *P* < 0.05). Restoration of Bmi1 expression did not affect the protein level of ERα (Figure 5B), and we therefore conclude that ERα is an upstream regulator of Bmi1, with no feedback regulation between ERα and Bmi1.
Figure 3: ERα suppresses EMT and cell stemness. A. Morphology of pEGFP-C1 and pEGFP-C1-ERα BT549 cells. B. Morphology of T47D cells transfected with control or ERα siRNA. C. Wound healing assay in pEGFP-C1 or pEGFP-C1-ERα BT549 cells. D–E. Migration and invasion assays of pEGFP-C1 and pEGFP-C1-ERα BT549 cells. Representative images of migrating (D) or invading (E) cells are shown. F. Representative images of mammosphere forming units (MFUs) observed in pEGFP-C1 and pEGFP-C1-ERα BT549 cells. Data are mean ± SEM (n = 3). G. FACS analysis of cell-surface markers CD44 and CD24 in pEGFP-C1 and pEGFP-C1-ERα BT549 cells. Data are mean ± SEM (n = 3). *P < 0.05 compared to pEGFP-C1 BT549 cells (Student’s t test). Magnification, 400 × (A–B, D–F); 100 × (C).
Figure 4: ERα expression reduces the metastasis of BT549 cells. A. H&E staining of metastatic nodules (red box) in mouse lung tissue. Magnification, 100× (upper); 200× (bottom). Bar = 50 μM. B. Number of mice with pulmonary nodules (n = 5).

Figure 5: Restoration of Bmi1 expression reverts ERα-suppressed EMT and stemness. A. Western blot analysis of Bmi1 protein in BT549 cells transfected with the indicated vectors. B. Western blot analysis of ERα, Bmi1 and E-cadherin protein levels in pEGFP-C1 or pEGFP-C1-ERα BT549 cells transiently transfected with the indicated vectors. C–D. Migration and invasion assays. Representative images of migrating (C) or invading cells (D) are shown. E. Morphology and quantification of mammospheres. Ctrl: pEGFP-C1 BT549 cells transiently transfected with control plasmid pcDNA3; ERα+Ctrl: pEGFP-C1-ERα BT549 cells transiently transfected with pcDNA3; ERα+Bmi1: pEGFP-C1-ERα BT549 cells transiently transfected with pcDNA3-BMI1. *P < 0.05, pEGFP-C1-ERα BT549 cells transfected with pcDNA3 (ERα+Ctrl) compared with pEGFP-C1 BT549 cells transfected with pcDNA3 (Ctrl). #P < 0.05, pEGFP-C1-ERα BT549 cells transfected with pcDNA3 (ERα+Ctrl) compared with pEGFP-C1-ERα BT549 cells transfected with pcDNA3 Bmi1 (ERα+Bmi1) (Student’s t test). Data are mean ± SEM (n = 3). Magnification, 400× (C–E).
**Bmi1 expression is negatively associated with ERα and E-cadherin levels in human breast cancer**

We further analyzed the mRNA levels of Bmi1 and E-cadherin in 58 human breast cancer samples. Bmi1 mRNA levels were significantly lower in ERα-positive than in ERα-negative tissues (Figure 6A; \( P < 0.05 \)), but E-cadherin mRNA levels were significantly higher in ERα-positive than in ERα-negative tissues (Figure 6B; \( P < 0.05 \)). Furthermore, immunohistochemistry staining for Bmi1, ERα and E-cadherin revealed that Bmi1 expression was inversely associated with ERα and E-cadherin levels in human breast cancer specimens in a significant manner (Table 1 and Figure 6C; \( P < 0.05 \)). We demonstrated an inverse linear correlation of ERα or E-cadherin expression, with Bmi1 expression, when the percentage of positively stained cells for each protein was determined in human breast cancer tissues (Figures 6D and 6E; \( P < 0.05 \)). These results support a model of ERα interacting with the Bmi1 locus, leading to the transcriptional repression of Bmi1 for the downregulation of E-cadherin.

**DISCUSSION**

Breast cancer is the most commonly diagnosed cancer in women and remains one of the leading causes of death [1]. Most breast cancer-related deaths occur after extensive metastasis, during which EMT endows cancer cells with increased migration and invasive capabilities. Since ERα has been shown to suppress EMT, efforts to elucidate the molecular mechanisms by which ERα regulates EMT, as well as inhibits stemness, in breast cancer are warranted in order to develop novel treatments for this common type of cancer. In the current study, we have demonstrated that ERα can suppress Bmi1 expression by transcriptional repression, and inhibit EMT and stemness in breast cancer through an ERα–Bmi1–E-cadherin pathway.

The presence of ERα represents a epithelial phenotype in the breast. Previous studies suggested that ERα upregulates several epithelial markers, such as E-cadherin, in breast cancer cells. In studying the role of Bmi1 in hormone-dependent and -independent breast cancer cells, we found that Bmi1 mRNA and protein levels were lower in ERα-positive than in ERα-negative cells. Furthermore, estrogen stimulation downregulated Bmi1 expression in hormone-dependent breast cancer cells, while in hormone-independent cells, Bmi1 was downregulated by ERα overexpression. When ERα expression was silenced by RNA interference, Bmi1 became upregulated. These findings suggest that ERα modulates the Bmi1 signaling pathway.

ERα has previously been shown to bind to the ERE/half-ERE region in the promoter of its downstream genes to regulate gene expression [33, 34]. For instance, ERα can repress Slug transcription by binding to the half-ERE element in the Slug promoter to regulate

| Table 1: Association of Bmi1, ERα and E-cadherin expression in human breast cancer tissues |
|------------------|------------------|------------------|
|                  | **n** | **Bmi1** | **P value** |
|                  |       | **Negative (%)** | **Positive (%)** |       |
| ERα              |       |                 |                 |       |
| Negative         | 26    | 13 (50.0)       | 13 (50.0)       | 0.003 |
| Positive         | 32    | 28 (87.5)       | 4 (12.5)        |       |
| E-cadherin       |       |                 |                 |       |
| Negative         | 28    | 16 (57.1)       | 12 (42.9)       | 0.043 |
| Positive         | 30    | 25 (83.3)       | 5 (16.7)        |       |
E-cadherin expression and EMT [2, 7]. Interestingly, the BMI1 promoter sequence includes a half-site ERE element (TGACC). Evidence that ERα directly binds to the TGACC site in the BMI1 promoter suggests that ERα-mediated Bmi1 suppression is indeed regulated at the transcriptional level. This conclusion is further supported by our dual luciferase reporter gene assay revealing increased/decreased activity of a BMI1 promoter-driven reporter gene in ERα silenced/overexpressing breast cancer cells. Our findings indicate that ERα suppresses Bmi1 expression by directly binding to the half-ERE site of the BMI1 promoter, thereby demonstrating a novel pathway by which ERα suppresses migration, invasion, and EMT in breast cancer cells, in addition to modulating Slug [2, 7] and NF-κB [6] pathways. Interestingly, a recent article by Wang et al. reported that ERα bound
to the promoter of Bmi1, as also identified by our study, suggesting that the ERs could transcriptionally modulate Bmi1 expression [35]. In contrast to our findings, however, Wang’s study found that Bmi1 expression was upregulated by the overexpression of ERα in another ERα-positive breast cancer cell line, MCF-7, while depletion of this protein caused a down-regulation of Bmi1. Whether this well-demonstrated, opposing regulation is cell line-specific needs to be investigated in future studies.

Song et al. showed that Bmi1 could inhibit phosphatase and tensin homologs, induce EMT and also regulate the self-renewal and differentiation of stem cells in human nasopharyngeal epithelial cells [21]. In pancreatic cancer, the EMT-activator, Zeb1, maintains stemness of cells, in part, through Bmi1 [22]. In our study, the elevated expression of ERα decreased the CD44high/CD24low cell population in breast cancer cells and inhibited the cells’ capacity to form mammospheres. Most importantly, restoring Bmi1 expression reversed the ERα-mediated suppression of EMT and stemness. The current findings highlight the critical role of Bmi1 in regulating both EMT and stemness in breast cancer cells. Although ERα expression represses the stemness of cancer cells, the underlying mechanism of ERα in regulating stemness has not been thoroughly explored. ERα may modulate the stemness of breast cancer cells by suppressing Bmi1 expression, and may therefore be considered an inhibitor of circulating and migrating cancer stem cells.

In summary, our results demonstrate that ERα can suppress EMT in human breast cancer cells through the transcriptional down-regulation of Bmi1 and its downstream genes. An inverse relationship between ERα and Bmi1 expression further supports the epithelial phenotype of ERα-positive tumors, or the mesenchymal phenotype of ERα-negative tumors, as being most likely regulated via the ERα–Bmi1–E-cadherin axis. Our findings provide a novel mechanistic insight into how ERα regulates EMT and may be of value in developing new biomarkers for the prognosis of breast cancer.

**MATERIALS AND METHODS**

**Cell lines, antibodies and plasmids**

BT474, T47D, MDA-MB-231, BT549 and SKBR3 breast cancer cell lines were purchased from the Committee on Type Culture Collection of the Chinese Academy of Science (Shanghai, China). The pEGFP-C1-ERα plasmid was purchased from Addgene (Cambridge, MA, USA), and pcDNA3-BMI1 plasmid was a gift from Dr. MH Yang (Institute of Clinical Medicine, National Yang-Ming University, Taipei City, Taiwan) [20]. A wild type BMI1 promoter (pXP2–BMI1–Luc1000) from −1023 to −1 was constructed and a mutant BMI1 promoter was generated by replacing a TGACC (−178–174) sequence in the wild type BMI1 promoter with a GACCC sequence. The following antibodies were used in this study: anti-E-cadherin (DAKO, Glostrup, Denmark; NCH-38), anti-Bmi1 (Abcam, Cambridge, UK; ab126783), anti-ERα (Santa Cruz Biotechnology, CA, USA; sc-543), anti-β-actin (Santa Cruz Biotechnology, Dallas, TX, USA; sc-4777), PE-conjugated anti-CD24 (BD Biosciences, Bedford, MA, USA; ML5), APC-conjugated anti-CD44 (BD Pharmigen, San Jose, CA, USA; G44–26), PE-conjugated mouse IgG (BD; G155–178), and APC-conjugated mouse IgG (BD; 27–35).

**Transfections**

We transfected 5 μg of the vectors, pEGFP-C1 or pEGFP-C1-ERα, containing full-length human ERα, into BT549 cells growing in 10-cm tissue culture plates using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Stable clones were selected and established by culturing in Dulbecco’s modified Eagle’s medium (DMEM) plus 10% fetal bovine serum (FBS) containing 0.8 mg/mL G418 (Merck & Co., Whitehouse Station, NJ, USA). To clarify the role of Bmi1, 5 μg of pcDNA3 or pcDNA3-BMI1 was transfected into pEGFP-C1-ERα stable cells growing in 10-cm tissue culture plates for 48 h. ERα siRNA and scrambled siRNA were synthesized by GenePharma Biotech (Shanghai, China). ERα siRNA-1 sequences were as follows [2]: 5’-CGAGUAUAGCUACCCAGAII-3′ (sense) and 5’-UCUGUAGGAUCAUACUCGGA-3′ (antisense); siRNA-2 sequences: 5’-AAGCUACGUUUGCUCUAACTT-3′ (sense) and 5’-GUUAGGAGCAAAACAGUACUUTT-3′ (antisense). Scrambled siRNA sequences were 5’-UUCUCCCAGCUUGCCGU-3′ (sense) and 5’-ACUGUACACGUUCCGGA-3′ (antisense). Transfection of siRNA involved the use of Lipofectamine 2000. Briefly, 5 or 50 nmol/L ERα siRNA or scrambled siRNA was transfected into cultured T47D cells (at 50% confluence) for 48 h.

**RNA isolation and RT-PCR**

TRIzol reagent (Invitrogen, Carlsbad, CA) was used to isolate total RNA from cultured cells or fresh-frozen breast cancer tissue. The PrimeScript RT Reagent Kit (Takara, Dalian, China) with random primers was used to synthesize cDNA. PCR reactions involved 2 μL cDNA and the ABI PRISM 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Expression was normalized to that of the β-actin housekeeping gene as an internal control. The primer sequences were for β-actin, 5′-AGCGAGCATCCCCAAAGTT-3′ (sense) and 5′-GGGCACGAAGGCTCATCATT-3′ (antisense); ERα, 5′-TGCTTCAGGCTACCATTATGGA-3′ (sense) and 5′-GGGCACGAAGGCTCATCATT-3′ (antisense); Bmi1, 5′-GCTGCCAATGGCTCTAATGAA-3′ (sense) and 5′-TGGCTGGACACATATAGTCGTT-3′ (antisense); and 5′-TGGCTGGACACATATAGTCGTT-3′ (antisense); and 5′-ACGUGACACGUUCCGUAACTT-3′ (sense) and 5′-GUUAGGAGCAAAACAGUACUUTT-3′ (antisense). Scrambled siRNA sequences were 5’-UUCUCCCAGCUUGCCGU-3′ (sense) and 5’-ACUGUACACGUUCCGGA-3′ (antisense). Transfection of siRNA involved the use of Lipofectamine 2000. Briefly, 5 or 50 nmol/L ERα siRNA or scrambled siRNA was transfected into cultured T47D cells (at 50% confluence) for 48 h.
Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as previously described [36]. In brief, T47D cells at 80% to 90% confluence growing in 10-cm dishes were treated with 1% formaldehyde for 10 min to cross-link proteins to DNA, and then sonicated four times for 10 sec by use of a sonicator with a microtip in a 1.5-mL tube. The resultant lysate underwent immunoprecipitation (IP) with 1 μg polyclonal anti-ERα antibody. Normal IgG was used as an IP control, and the supernatant was used as an input control. Immunoprecipitated complexes were collected by adding protein A/G-agarose/salmon sperm DNA beads and incubating samples for 2 h at 4°C. The beads were then treated with RNase A (50 μg/mL) and proteinase K. DNA was extracted with phenol/chloroform and co-precipitated with glycogen, dissolved in 25 μL TE buffer, and subjected to PCR amplification for ERα binding sites in the BMI1 promoter using specific primers: BMI1–1, 5′-CGGGCTGTACATACCGACAC-3′ (sense), and 5′-GGAAGCTGCACACGGCTC-3′ (antisense); BMI1–2, GCAGAGGAAAACCAGAAACG-3′ (sense) and 5′-TGGGCAGTATCTTTCCCTCTT-3′ (antisense). The acquired DNA was resolved on a 2% agarose gel and stained with Goldview.

Electrophoretic mobility shift assay (EMSA)

Oligonucleotides

(5′-AGCACGTGACCCGCTGGG-3′) containing putative binding sequences of ERα were labeled at the 3′-end with biotin and incubated with nuclear extracts harvested from BT549 cells transfected with pEGFP-C1-ERα. The binding reaction (20 min at room temperature) was carried out in a final volume of 20 μL containing the biotin-labeled probe (20 fmol), with nuclear extract according to the LightShift Chemiluminescent EMSA Kit (Beyotime Biotech, Haimen, China) manufacturer’s protocol. In supershift experiments, the nuclear extract was preincubated with anti-ERα antibody for 30 min on ice. In non-specific binding assays, poly (dI:dC), nonspecific antibodies, including normal IgG or anti-Twist1 antibody, were added instead of anti-ERα antibody. In the competition assay, excess amounts of unlabeled competitors, including putative binding sequences and mutant sequences (5′-AGCACGGACCCGCTGGG-3′), were added before the labeled probes. After binding, the samples were separated on 6% non-denaturing polyacrylamide gels and visualized by enhanced chemiluminescence.

Cell migration and invasion assays

A modified cell migration assay was performed using BT549 cells stably transfected with pEGFP-C1 or pEGFP-C1-ERα cells as described previously [26]. An 8-μm pore-size Boyden chamber (BD) was used for in vitro migration and invasion assays. Cells (5 × 10³) incubated in DMEM supplemented with 1% bovine serum albumin were plated in the upper chamber, and 10% fetal bovine serum was added to DMEM in the lower chamber as a chemoattractant. A BD BioCoat Matrigel Invasion Chamber (BD) was used for the invasion assay. After 24 h (migration assay) or 72 h (invasion assay), cells on the upper side of the filter were removed, and cells that remained adherent to the underside of membrane were fixed in 4% formaldehyde and stained with crystal violet dye. Five random fields per membrane were photographed with the use of a BX51 microscope (Olympus, Tokyo, Japan) at ×400 magnification. The cells were counted and the mean number of the five fields was calculated to obtain a representative number of cells that had migrated/invaded across the membrane. Three independent experiments were performed for each assay.

Wound healing assay

BT549 cells stably transfected with pEGFP-C1 or pEGFP-C1-ERα were seeded in 6-well plates at a density of 3 × 10⁴ cells/well. Cells were then serum-starved for 24 h, and a linear wound was created in the confluent monolayer by use of a pipette tip. Wound healing was photographed at 24 h intervals. Experiments were done in triplicate, and three random fields of each well were recorded.

Flow-cytometric analysis of CD44 and CD24 expression

To analyze CD44 and CD24 expression, cells were trypsinized and suspended in phosphate buffered saline (PBS), plus 1% fetal bovine serum, at a density of 10⁵ cells/100 μL. Cells were incubated at 4°C for 40 min with PE-conjugated anti-CD44 antibody and APC-conjugated anti-CD24 antibody, or their respective isotype controls, at concentrations recommended by the manufacturer. Labeled cells were washed twice with PBS, then fixed in PBS containing 1% paraformaldehyde and analyzed with the use of a FACSCalibur flow cytometer (BD).

Mammosphere assay

Single cells were plated at a density of 1,000 cells/mL in 96-well ultralow attachment plates (Corning, New York, USA). Cells were grown in serum-free DMEM/F12 supplemented with B27 (1:50, Invitrogen), 20 ng/mL endothelial growth factor, 20 ng/mL basic fibroblast growth factor and 4 μg/mL heparin (Sigma, St. Louis, MO, USA). Cells were cultured for 7 d, and mammospheres with >50 μm diameter were counted.
Immunohistochemistry (IHC) of human breast cancer tissues

Human breast cancer specimens were obtained from 58 patients who underwent breast cancer surgery at the Cancer Hospital of Shantou University Medical College, China between 2010 and 2011. Written informed consent was obtained from each patient, and the study was approved by the Hospital Research Ethics Committee.

Serial formalin-fixed and paraffin-embedded tissues were sectioned at a 4 μm thickness, deparaffinized, and rehydrated in gradients of high percentage ethanol to distilled water. For quenching endogenous peroxidase activity, sections were immersed in 3% hydrogen peroxide for 15 min at room temperature. Antigen retrieval involved boiling in 10 mM sodium citrate buffer (pH 6) for 3 min in a pressure cooker, followed by cooling to room temperature. Sections were then incubated with the primary antibody at 4ºC overnight, washed three times in PBS for 5 min, and incubated with horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG antibody (ZSGB-Bio, Beijing, China) at room temperature for 30 min, followed by 3, 3′-diaminobenzidine tetra-hydrochloride (DAB) staining. Sections were lightly counterstained with hematoxylin.

The expression of ERα, Bmi1 and E-cadherin was detected using specific antibodies, with normal mouse or rabbit IgG (Santa Cruz Biotechnology) used as the negative control. Cases were considered as positive for ERα if nuclear immunoreactivity was present in ≥10% of tumor cells. For nuclear Bmi1 staining, the percentage of stained cells was categorized into 0 to 3+ as previously described [20]: 0, no staining; 1+, 1–25%; 2+, 25–50%; and 3+, > 50% nuclear staining. Only 3+ was considered as a positive IHC result. E-cadherin expression was interpreted as either normal (strong) or aberrant (reduced or absent). Aberrant staining was defined as either negative staining or <50% membranous staining of the population of cells examined. Normal staining was defined as ≥50% membranous staining of the cancer cells [37]. The percent of positively stained cells was evaluated, in the same section of tissue, to analyze the correlation between Bmi1 and ERα or E-cadherin.

In vivo metastasis in mice

Eight- to 10-week-old female BALB/C nude mice were intravenously injected, via the tail vein, with 5 × 10^7 pEGFP-C1 or pEGFP-C1-ERα stable cells in 0.1 mL PBS (5 mice for each group). Six weeks after injection, mice were euthanized to grossly detect metastases. The visceral organs, such as lung and liver, were fixed with formalin, then paraffin-embedded and stained with hematoxylin and cosin (H&E). Metastatic lesions were evaluated by microscopic examination. The animal study was approved by the Institutional Animal Care and Use Committee of Shantou University Medical College.

Statistical analysis

Statistical analysis was performed using SPSS 16.0 (SPSS Inc., Chicago, IL, USA). Differences among variables were assessed by y2 analysis, Spearman’s Rank Correlation Test or 2-tailed Student’s t tests. Data were presented as the mean ± SEM unless otherwise indicated. Two-sided P < 0.05 was considered statistically significant. Each experiment was performed at least three times.

ACKNOWLEDGMENTS

This work is partly supported by the funds from Major State Basic Research Development Program of China (No.2011CB707705), the Natural Science Foundation Committee (No.31271068, 81302331), and the Guangdong Provincial Key Laboratory on Breast Cancer Diagnosis and Treatment Research.

CONFLICTS OF INTERESTS

None of the authors have any financial or personal relationships with other people or organizations that could inappropriately influence this research.

REFERENCE


34. Petz LN, Nardulli AM. Sp1 binding sites and an estrogen response element half-site are involved in regulation of the human progesterone receptor A promoter. Molecular endocrinology. 2000; 14:972–985.

