PBX1 Participated in Estrogen Mediated Bladder Cancer Progression and Chemo-Resistance through Affecting Estrogen Receptors

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Abstract

Bladder cancer (BCa) is a common cancer associated with high morbidity and mortality worldwide. Pre-B-cell leukemia transcription factor 1 (PBX1) has been reported to be involved in tumor progression. The aim of the study was to explore the specific role of PBX1 in BCa and its underlying mechanisms. The relative expressions of PBX1 in muscle-invasive BCa tissues and cell lines were analyzed through RT-qPCR and Western blotting. Kaplan–Meier analysis was used to analyze the relationship between PBX1 levels and survival status. Co-immunoprecipitation (CO-IP) and chromatin immunoprecipitation (ChIP)-qPCR assay were adopted to verify the interaction between PBX1 and Estrogen receptors (ERs), and explore the ER-dependent genes transcription. The results shown that PBX1 was upregulated in invasive BCa patients and BCa cells, positively associated with tumor size, lymph node metastasis, distant metastasis and poorer survival status. Overexpression of PBX1 promoted cell growth, invasion, epithelial-mesenchymal transition (EMT) process and cisplatin resistance in BCa cells, while the silence of PBX1 showed opposite effects. Further, PBX1 interacted with Estrogen receptors (ERs) and was required for the ER function. Overexpression of PBX1 aggravated the tumor-promoting effect of estrogen on BCa cells, while partially suppressed the inhibitory effects of ER antagonist AZD9496 on BCa cells. In summary, this study revealed that PBX1 participated in estrogen mediated BCa progression and chemo-resistance through binding and activating estrogen receptors. Hence, PBX1 may serve as a potential prognostic and therapeutic target for BCa treatment.

Introduction

Bladder cancer (BCa) is a common cancer worldwide and associated with the high rates of morbidity and mortality [1]. Data from the World Health Organization (WHO) showed that there were about 549,393 new cases diagnosed and 199,922 deaths in the world in 2018 [2]. BCa develops into two distinct types: non-muscle-invasive papillary tumor (70–80%) and non-papillary (solid) muscle-invasive tumor (20–30%) [1]. Commonly, patients with muscle-invasive bladder tumor accompanied with higher risks of metastasis and poor prognosis [3]. The five-year overall survival rate of muscle-invasive BCa patients is only 60 % due to the distant metastasis and high recurrence rates [4]. Therefore, it is of great clinical significance to explore the molecular mechanism that drives the progression of muscle-invasive BCa, which will help to develop more effective anticancer therapies.

PBX1 (Pre-B-cell leukemia transcription factor 1), a stem cell reprogramming factor, has a crucial role in the hematopoiesis and development of pancreas, spleen, kidney, urogenital tract, and skeleton [5–7]. It was first reported as a translocated proto-oncogene and exhibited an oncogenic potential in leukemia [8]. In addition, PBX1 was upregulated during the progressions of ovarian, melanoma, prostate, and breast cancers [9–13]. High expression level of PBX1 was negatively related to the survival of ovarian cancer patients, post-chemotherapy [9]. Enhanced PBX1 transcription could promote cell proliferation and metastasis in ER α -positive breast cancer and renal clear cell carcinoma [14, 15]. In addition, PBX1 was a key gene responsible for the bladder and kidney development, which was upregulated in fetal period and declined in adults, and the haploinsufficiency of PBX1 was regarded as a crucial factor causing

congenital anomalies of the kidney and urinary tract [16]. However, the expression pattern and specific role of PBX1 in BCa remain unclear.

Recent evidence showed that women were more likely to develop an advanced tumor stages of BCa with nodal metastasis, although they had a lower BCa incidence compared with man [17]. Estrogen receptors (ERs) were found expressed in BCa tissues and cell lines [18, 19]. Estrogen receptor α (ER α) and Estrogen receptor β (ER β) are two major isoforms of ER and was reported to play an important role in BCa initiation and growth. ER β could promote the growth and invasion ability of BCa cells [20], and induce cisplatin resistance in BCa [21]. ER α and ER β are located in the nucleus and mediate the genotype effect of estrogen through regulating the transcriptions of downstream target genes, such as *MYC*, *cFOS*, *EGR3* and *GREB1* [22, 23]. Higher expression of PBX1 was reported to be associated with the poor prognosis of ER-positive breast cancer patients [24]. Meanwhile, PBX1 was increased in the tissues of ER α -positive breast cancer patients during the metastatic progression [15]. Recent studies revealed that PBX1 was required for ER activity, and involved in the activation of ER [22, 23].

In this study, we examined the expression of PBX1 in BCa patient tissues and cells, and its association with the clinicopathological characteristics of BCa patients. Next, the effects of PBX1 alteration on cell proliferation, apoptosis, and migration in BCa cells were measured. Furthermore, the underlying mechanism of PBX1 on the BCa cells was explored based on ER activation. Our study may provide a novel evidence for BCa therapy and prognosis.

Materials And Methods

Patients and samples collection

A total of 85 muscle-invasive BCa patients who underwent curative resection in Yantai Affiliated Hospital of Binzhou Medical University from 2012 to 2013 were recruited in this study. In line with the criteria of WHO, the specimens were confirmed by pathological specialists. The patients included were anonymous and had suffered cisplatin (CDDP) therapy before the surgery. According to their outcomes, relapse or not, we classified them into CDDP resistant and sensitive groups. The BCa tumor tissues and un-paired non-tumor bladder tissues were harvested for analyzing the mRNA expression levels of PBX1. Five paired tumor tissues and non-tumor tissues were adopted to measure the protein expression of PBX1. Overall survival (OS) analysis was performed using the Kaplan-Meier method. This study was approved by the Institutional Review Board of Yantai Affiliated Hospital of Binzhou Medical University. Also, the whole experiment was under the guideline of Declaration of Helsinki. Informed consents were obtained from the corresponding patients before surgery.

Cell lines

BCa cell lines (J82, EJ, T24, UMUC3 and 5637) and a Human bladder epithelial immortalized cells (SV-HUC-1) were obtained from Chinese Academy of Sciences Committee on Type Culture Collection cell bank (Shanghai, China). Cells were maintained in DMEM culture medium (GIBCO BRL, Gaithersburg, MD)

with 10 % fetal bovine serum (FBS, Invitrogen, Carlsbad, CA, USA). In line with the method described in previous study, the CDDP resistant T24 (T24 CR) and UMUC3 (UMUC3 CR) cells were established [25].

RNA extraction and Realtime quantitative PCR (RT-qPCR)

Total RNAs were isolated from BCa tissues and cells by using a Trizol kit (Qiagen, Valencia, CA). RT-qPCR was performed as described previously to examine mRNA expressions [26]. GAPDH was used as an internal control and each reaction was performed in triplicate. The primers used in this study was provided in Table 1.

Cell transfection and treatments

Full-length complementary DNA for PBX1 was cloned from the genomic DNA of BCa cells and ligated onto the sequences of pcDNA3.1 (+) vector which was purchased from Realgene (Nanjing, China). PBX1 siRNA (si-PBX1) and negative control siRNA (si-NC) were constructed by Genepharma Technology (Shanghai, China). Lipofectamine 3000 (Invitrogen, USA) was adopted for the transfection assay following the manufacturers' protocol. The transfection dose of overexpression vector and siRNAs were 0.5 µg and 50 nM, respectively, and transfection procedure lasted for 48 h.

CDDP drugs were purchased from MedChemExpress (Shanghai, China) and utilized in line with the protocol of manufacturers. The cultured cells were treated with different doses of CDDP and assessed cell viability at 24 h by using CCK-8 method. Finally, the drug sensitivity images and IC50 values for CDDP in every group were obtained on GraphPad Prism 8.0 software (GraphPad Software Inc., San Diego, USA).

CCK-8 assay

Cell viability was analyzed using a Cell Counting Kit-8 (CCK-8, Dojindo Laboratories, Kumamoto, Japan). In brief, cells were planted into 96-microwell plates with a density of 2000 cells/well. After transfection with overexpression vectors or siRNAs for 24, 48 and 72 h. The optical absorbance at 450 nm was detected using a plate reader (Thermo Fisher Scientific).

Colony formation assay

Cells were planted into 24-well plates with a density of 200 cells/well. Culture medium was replaced every two days. After cultured for two weeks, colonies were fixed and permeated using acetone/methanol (1:1, v/v) for 15 min. Then colonies were stained with 0.5% (w/v) crystal violet (Beyotime Biotechnology, Shanghai, China) for 15 min. Following wash with distilled water and dry at room temperature, the colonies from five randomly selected fields were captured under an inverted phase contrast microscope, and colonies numbers were counted.

Cell invasion assay

Cell invasion ability was measured using a Transwell chamber (Corning, Corning, NY, USA) containing polycarbonate filters with 8.0 µm pore size. Cells (1×10^5) and mixed with serum free medium. The cultured cells were seeded into the top chamber with a Matrigel-coated membrane. Complete medium

was added into the lower chamber. After several hours of incubation at 37°C, cells that invaded to the lower chamber were fixed and stained in 20% methanol and dye solution containing 0.1% crystal violet. The number of cells that had invaded was counted using an inverted microscope (Olympus, Tokyo, Japan).

Western blotting

Total proteins were extracted and quantified using the RIPA lysis buffer and a BCA Protein Assay kit (Beyotime, Shanghai, China). Then, proteins were separated by electrophoresis in 10% SDS-PAGE and transferred onto nitrocellulose membranes. The membranes were blocked with 5% skim milk and incubated with specific primary antibodies (4°C, overnight) and secondary antibodies (2 h, room temperature), in order. Image J Software was adopted for data analysis after developing with western ECL substrate (Share-bio, Shanghai, China).

Co-immunoprecipitation (Co-IP) assay

For Co-IP assay, protein samples were incubated with anti-PBX1 (1:200) agarose slurry (Sigma, USA) or control IgG for 2 h at 4°C [27]. The beads were collected by a centrifugation at 3000 rpm for 5 min. Then, the precipitated proteins were subjected to WB.

Chromatin immunoprecipitation (ChIP)

ChIP was performed using a ChIP assay kit (Active Motif, USA). T24 cells were crosslinked with 1% formaldehyde and then the chromatin was sheared by micrococcal nuclease. The digested chromatin was incubated with ER antibody and IgG overnight at 4°C. After the immunoprecipitation, protein A/G plus agarose was added. Then, precipitates were washed, and purified DNA was subjected to qPCR detection. The specific primers used in this study were provided in Table 1.

Data Analysis

All data were shown as the mean $\pm$ standard deviation (SD). Data analysis were performed using SPSS 18.0 software (IBM, NY, USA) and GraphPad Prism Version 7 (GraphPad Software). One-way ANOVA and *t*-test were performed to analyze the comparison between multiple groups and two groups. Overall survival (OS) analysis was performed using the Kaplan-Meier method. Statistical significance was defined when $p < 0.05$.

Results

PBX1 was upregulated in BCa patients and cells

The expression pattern of PBX1 in BCa tumor tissues ($n = 85$) and paired non-tumor tissues ($n = 53$) were examined through RT-qPCR. As shown in Fig. 1A, the expression of PBX1 mRNA was significantly upregulated in BCa tumor tissues ($p < 0.01$). Kaplan–Meier analysis indicated that BCa patients with higher expression of PBX1 had significantly lower overall survival rate and outcome ($p = 0.046$, Fig. 1B).

The association between PBX1 expression and clinicopathologic characteristics of BCa patients were summarized in Table 2. Besides, the results showed that the level of PBX1 was positively associated with tumor size ($p = 0.0326$), lymph node metastasis ($p = 0.0348$) and distant metastasis ($p = 0.0188$). Similarly, the protein expression of PBX1 was upregulated in BCa tissues compared with the normal ones (Fig. 1C). We compared the expression levels of PBX1 in SV-HUC-1 cells (a normal human bladder cell line) and BCa cell lines (J82, UMUC3, 5637, EJ, and T24) through Western blotting. The results indicated that BCa cells showed significantly higher expression of PBX1 (Fig. 1D).

Effect of PBX1 alteration on the growth and apoptosis of BCa cells

Overexpression vector of PBX1, and siRNA against PBX1 were transfected into T24 and UMUC3 cell lines. As shown in Fig. 2A-C, the expression of PBX1 mRNA and protein were markedly increased in ov-PBX1 transfected T24 and UMUC3 cell lines, and decreased in si-PBX1 transfected T24 and UMUC3 cell lines (Fig. 2A-C). Next, the results showed that the overexpression of PBX1 significantly promoted the cell proliferation and colony formation ability of T24 and UMUC3 cell lines (Fig. 2D-G). Meanwhile, the silence of PBX1 suppressed the growth of T24 and UMUC3 cells (Fig. 2D-G), and induced cell apoptosis in T24 and UMUC3 cells, while PBX1 overexpression showed no effect on cell apoptosis (Fig. 2H-J).

Effect of PBX1 alteration on the invasion and epithelial-mesenchymal transition (EMT) activity of BCa cells

Next, overexpression of PBX1 was found to greatly promote the invasion ability of T24 and UMUC3 cells, while inhibition of PBX1 has the opposite effect (Fig. 3A and B). EMT biomarkers, E-cadherin, N-cadherin, Vimentin, fibronectin and Snail, were analyzed through Western blotting. The results showed that the expression of E-cadherin was decreased in T24 cells after overexpression of PBX1 (Fig. 3C). In contrast, the expressions of N-cadherin, Vimentin and fibronectin were markedly increased in T24 cells following upregulating PBX1, while si-PBX1 showed the opposite effects on the expressions of EMT biomarkers (Fig. 3C). However, the expression of Snail did not affect by PBX1 alterations in T24 cells (Fig. 3C).

Pbx1 Regulated Chemo-resistance Of Bca Cells

To illustrate the correlation between PBX1 alteration and CDDP resistance of BCa, we first evaluated the expression profile of PBX1 in patients' tissues. As shown in Fig. 4A, PBX1 expression level was dramatically elevated in CDDP-resistant BCa tissues. Compared with normal T24 and UMUC3 cells, the resistant type of them presented significant upregulation in PBX1 expression (Fig. 4B). Also, we found that PBX1 silence decreased the promoted CDDP resistances and increased IC₅₀ values in both T24 CR and UMUC3 CR cells (Fig. 4C and 4D). The expressions biomarkers of drug-resistance, P-gp and MRP1 [28], were increased in CR cells and depressed by PBX1 inhibition (Fig. 4E).

PBX1 mediated the ER function

It has been reported that targeting to the corresponding pathway of anti-estrogen strategy emerged as a novel promising preclinical way for BCa [29]. Hence, our study explored the role of PBX1 in regulating ER function via measuring the ER-dependent gene transcriptions in T24 cells. We found that PBX1 overexpression or inhibition both made no significant effect on ER α and ER β expression (Fig. 5A). The Co-IP assay showed that endogenous PBX1 interacted with endogenous ER α and ER β in T24 cells (Fig. 5B). Previous studies indicated that ER could bind with the enhancer region of *EGR3*, *cFOS* and myelocytomatosis proto-oncogene (*MYC*), promoted their transcription [22]. In this study, the silence of PBX1 suppressed the binding of ER to the enhancer of *EGR3*, *cFOS* and *MYC* in T24 cells (Fig. 5C). Meanwhile, inhibiting PBX1 suppressed the transcription of *EGR3*, *cFOS* and *MYC* induced by estrogen (E2) (Fig. 5D). Next, we found that the overexpression of PBX1 facilitated the binding of ER in the enhancer region of *EGR3*, *cFOS* and *MYC* and promoted their transcription (Fig. 6A and B). Estrogen (E2) further promoted the transcriptions of *EGR3*, *cFOS* and *MYC* in ov-PBX1 transfected T24 cells (Fig. 6B). These results suggested that PBX1 was required for ER function.

Effect of PBX1 overexpression on E2 or ER antagonist treated T24 cells

As shown in Fig. 7, estrogen (E2) treatment significantly promoted the proliferation, colony formation, cell invasion and CDDP resistance of T24 cells, while ER antagonist, AZD9496, effectively suppressed the proliferation, colony formation, and cell invasion of T24 cells, and induced cell apoptosis. Next, the results showed that the silence of PBX1 suppressed the promoted effects of E2 on T24 cell proliferation, colony formation and invasion, while overexpression of PBX1 facilitated the promotion of E2 on T24 cells and partially suppressed the inhibitory effect of AZD9496 on T24 cells (Fig. 7A-D).

Discussion

In this study, we found that PBX1 was upregulated in tumor tissues of muscle-invasive BCa patients and BCa cell lines. Meanwhile, high expression level of PBX1 was positively related to tumor size, lymph node metastasis, distant metastasis and poorer survival status of muscle-invasive BCa patients. Besides, PBX1 mediated the growth, EMT progress, invasion ability and chemo-resistance of BCa cells. EMT is a necessary process in cancer metastasis, it promoted the BCa metastasis and recurrence [30]. Gugnoni *et al.* indicated that inducing the attenuation of EMT progress by CDH6 down-regulation dramatically depressed the metastatic behavior of papillary thyroid carcinomas [31]. Another study demonstrated that tumor-associated neutrophils secreted IL-17a and stimulated the EMT feature of gastric cancer cells leading to tumor malignant progression [32]. Consistent to our findings, it has been reported that PBX1 was involved in the EMT process of esophageal cancer and non-small cell lung adenocarcinoma via collaboration with ZEB2 and PREP1 [33, 34]. These results suggested that PBX1 may participated in BCa progression and may serve as a new prognostic and therapeutic target.

Previous studies revealed that PBX1 interacted with ER and promoted ER activation in breast cancer inducing the promoted metastatic progression in ER α -positive breast cancer patients [22, 23]. In our study, PBX1 showed no obvious effect on the expression of ER α and ER β in T24 cells, but interacted with both ER α and ER β . Deeply consulting the regulatory mechanism, we found that the inhibition of PBX1 suppressed the recruitment of ER to the enhancer of *EGR3*, *cFOS* and *MYC* in T24 cells. Evidence indicated that *EGR3*, *cFOS* and *MYC* were served as the target of ER transcription [35, 23]. In addition, *cFOS* and *MYC* were discovered to be oncogenes during BCa development, involving the regulations of cell proliferation, differentiation and invasion [36, 37]. These results indicated that PBX1 was required for the post-transcriptional regulation of ER activity in BCa cells.

It is convinced that BCa incidence was higher in man than in women, but female BCa patients exhibited advanced TNM stage, shorter survival time and poor prognosis [17, 38, 39]. Accumulated evidence indicated that estrogens promoted BCa tumorigenesis [40, 41]. The function of estrogens was mediated by its multiple receptors, including ER α and ER β [38]. The agonists of ER α and ER β , estradiol, enhanced BCa cell proliferation [20, 42]. Meanwhile, antiestrogens suppressed BCa cell growth, *in vitro* and *in vivo* [43, 44]. Consistent with previous studies, our study found that estrogen promoted cell proliferation and migration, while ER antagonist AZD9496 suppressed cell proliferation and migration, induced cell apoptosis in T24 cells. Besides, our study found that PBX1 aggravated estrogen-induced cell growth and invasion, while partially weakened the inhibitory effect of ER antagonist on cell growth and invasion of BCa cells. This may be due to the carcinogenesis of estrogens was depended on its receptors, while PBX1 was required for the ER activity in BCa cells.

CDDP is a largely used tumor chemotherapy drug in clinic that exerts anti-cancer activity by formatting inter- and intra-strand DNA adducts [45, 46]. Research data presented that 50% ~ 70% patients received therapeutic responses after CDDP-based treatment [47]. However, a tough issue occurred in the following therapy progression, in which tumor acquired a resistance to CDDP causing a high possibility of relapse [48]. Deeply efforts devoting on the potential mechanism of chemo-resistance might hold a great importance for solving the bottleneck of BCa therapy. Our research indicated that PBX1 was associated with the CDDP resistance of BCa patients and knockdown of PBX1 effectively promoted drug sensitivity of BCa cells via affecting ER signaling. Previously, PBX1 had been proved to be involved in the development of ovarian cancer chemo-resistance [49]. Takuro et al. discovered that tamoxifen modulated estrogen-mediated ER signaling pathway to enhance the drug sensitivity of BCa cells to CDDP [50], which greatly supported our findings.

PBX1 was upregulated in tumor tissues of muscle-invasive BCa patients and associated with tumor size, lymph node metastasis, distant metastasis and poorer survival status. PBX1 mediated BCa cell growth, EMT processes, invasion, cell apoptosis and CDDP resistance. In addition, PBX1 interacted with ER α and ER β , and was required for ER function. Overexpression of PBX1 aggravated the tumor-promoting effect of E2 on T24 cells and partially suppressed the inhibitory effect of AZD9496. Hence, it is suggested that PBX1 participated in BCa progression and chemo-resistance through binding and activation estrogen receptors. This study provided a new therapeutic target for muscle-invasive BCa.

Declarations

Funding:

None

Compliance with ethical standards

Conflict of Interest The authors declare that they have no conflict of interest.

Ethical approval All the patients, diagnosed and treated at Yantai Affiliated Hospital of Binzhou Medical University, provided written informed consents. The whole study conformed to the Helsinki Declaration and the Health Guide for the Care and Use of Laboratory Animals (National Institutes), and obtained approvals the Institutional Research Ethic Committee and the Animal Ethical Committee of Yantai Affiliated Hospital of Binzhou Medical University.

Informed consent Informed consents were obtained from every patient included in this study.

Data Availability The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author contributions Yang Zhao and Yinxu Wanwrote the main manuscript text, Songlin Liu, Shuhang Li and Jizhong Che prepared figures 1-3. Gang Zhang,Yankai Xu and Aimin Tianprepared figures 4. All authors reviewed the manuscript.

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Figures

Figure 1

PBX1 was high expressed in BCa patients and cell lines, and related to poor prognosis. A. The mRNA expression of PBX1 was measured through RT-qPCR. T: muscle-invasive BCa tissues (n=85); N: un-paired non-tumor bladder tissues (n=53), ** P < 0.01. B. Kaplan–Meier survival curves of BCa patients with high and low PBX1 expression value (Values are expressed as median), ** P < 0.01. C. Five pairs of BCa samples were subject to Western blotting analysis of PBX1 expression. T1-T5: bladder tumor tissues; C1-C5: paired non-tumor bladder tissues. **P < 0.01. D. The expression of PBX1 protein in normal bladder cell

line (SV-HUC-1) and BCa cell lines were detected by Western blotting. ** $P < 0.01$ and *** $P < 0.005$ compared with SV-HUC-1 cells; ## $P < 0.01$ compared with J82 cells.

Figure 2

Effect of PBX1 overexpression and inhibition on the growth, and apoptosis of BCa cells. A-C. Transfection efficiency of ov-PBX1 and si-PBX1 in T24 and UMUC3 cells were detected by RT-qPCR and Western blotting. ov-PBX1: overexpression vectors of PBX1; si-PBX1: siRNA of PBX1. D-G. Cell proliferation was

detected by CCK-8 assay kit (D and E) and colony formation assay (F and G). H-J. Cell apoptosis was detected by the Annexin V/PI assay. In A-G, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ compared with ov-NC group; # $P < 0.05$ and ## $P < 0.01$ compared with si-NC group. In H-J, * $P < 0.05$ and ** $P < 0.01$ compared with si-NC group.

Figure 3

The effect of PBX1 on the migration and EMT activity of BCa cells. A and B. Cell invasion ability of T24 and UMUC3 cells were measured through Transwell assay. C. The expression of EMT biomarkers E-cadherin, N-cadherin, Vimentin, fibronectin and Snail in T24 cells were detected through Western blotting. ** $P < 0.01$ compared with ov-NC group; # $P < 0.05$ and ## $P < 0.01$ compared with si-NC group.

Figure 4

Inhibition of PBX1 suppressed CDDP resistance of BCa cells. A and B. The expression levels of PBX1 were measured by RT-qPCR in non-resistant and resistant BCa patients' tissues, as well as BCa cells. In A, ** $P < 0.01$; in B, ** $P < 0.01$ versus SV-HUC-1, # $P < 0.01$ versus T24, && $P < 0.01$ versus UMUC3. C and D. Cell viability of normal and CDDP resistant BCa cells was evaluated by CCK-8 methods at 24 h; ** $P <$

0.01; ## $P < 0.01$ versus CDDP + si-NC group. E. The protein expressions of P-gp and MRP1 were detected by western blotting assay.

Figure 5

Inhibition of PBX1 suppressed the ER-dependent transcription. A. The expression levels of ERα and ERβ were measured by RT-qPCR in T24 cells after PBX1 overexpression or inhibition. B. CO-IP was performed in T24 cells. IgG, immunoglobulin G. C. ER occupancy in the enhancer of EGR3, cFOS and MYC in T24

cells was measured by ChIP-qPCR after knockdown of PBX1. Values were expressed as fold enrichment (the mean percentage of input enrichment of the candidate gene/a control gene). D. The mRNA levels of EGR3, cFOS and MYC were measured by RT-qPCR in T24 cells. Cells were maintained in estrogen-free media for three days, and then treated by DMSO or E2 (estrogen; 100 μ M) for 24 hours. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ versus si-NC group; ### $P < 0.005$.

Figure 6

Overexpression of PBX1 promoted the ER-dependent transcription. A. ChIP-qPCR assay of ER and control IgG binding at the candidate target genes in T24 cells after overexpression of PBX1. B. The expression of EGR3, cFOS and MYC in T24 cells were measured through RT-qPCR. ** $P < 0.01$ and *** $P < 0.005$ compared with ov-NC group; ### $P < 0.005$.

Figure 7

Effect of PBX1 overexpression or inhibition on E2 or ER antagonist treated T24 cells. Cell proliferation (A), colony formation (B), cell apoptosis (C and D) and cell invasion (E) of T24 cells were measured. Cells were treated with E2 (100 μM) or ER antagonist AZD9496 (100 nM) for 24 h upon PBX1 overexpression or inhibition. F. IC50 values of cultured cells were evaluated. In A-E, * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.005$ compared with the control group; # $P < 0.05$ and ## $P < 0.01$ compared with E2 group; & $P < 0.05$

compared with AZD9496 group. In F, ** $P < 0.01$ compared with T24 group; # $P < 0.05$ and ## $P < 0.01$ compared with untreated T24 CR group; & $P < 0.05$ compared with E2 group; \$\$ $P < 0.01$ compared with ADZ9496 group.