Biochemical and Biophysical Research Communications 457 (2015) 404–411

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Differential effects of estrogen-dependent transactivation vs. transrepression by the estrogen receptor on invasiveness of HER2 overexpressing breast cancer cells

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ARTICLE INFO

Article history:
Received 16 December 2014
Available online 9 January 2015

Keywords:
Estrogen receptor
Tamoxifen
Anti-estrogens
Invasiveness
Breast cancer
HER2

ABSTRACT

Estrogen (E_2) supports breast cancer cell growth but suppresses invasiveness and both actions are antagonized by anti-estrogens. As a consequence, anti-estrogen treatment may increase the invasive potential of estrogen receptor (ER)+ tumor cell sub-populations that are endocrine resistant due to HER2 amplification. Either transactivation or transrepression by E_2/ER could lead to both up- and down-regulation of many genes. Inhibition of the transactivation function of ER is adequate to inhibit E_2-dependent growth. However, the impact of inhibiting E_2-dependent transactivation vs. transrepression by ER on regulation of invasiveness by E_2 is less clear. Here we dissect the roles of ER-mediated trans-activation and transrepression in the regulation of invasiveness of ER+/HER2+ breast cancer cells by E_2. Knocking down the general ER co-activators CBP and p300 prevented activation by E_2 of its classical target genes but did not interfere with the ability of E_2 to repress its direct target genes known to support invasiveness and tumor progression; there was also no effect on invasiveness or the ability of E_2 to regulate invasiveness. On the other hand, overexpression of a co-repressor binding site mutant of ER (L372R) prevented E_2-dependent transrepression but not transactivation. The mutant ER abrogated the ability of E_2 to suppress invasiveness. E_2 can partially down-regulate HER2 but knocking down HER2 below E_2-regulated levels did not affect invasiveness or the ability of E_2 to regulate invasiveness, although it did inhibit growth. Therefore, in ER+/HER2+ cells, the E_2-dependent transrepression by ER rather than its transactivation function is critical for regulation of invasiveness and this is independent of HER2 regulation by E_2. The findings suggest that selective inhibitors of transactivation by ER may be more beneficial in reducing tumor progression than conventional anti-estrogens that also antagonize E_2-dependent transrepression.

1. Introduction

Estrogen receptor type α (ER)-positive breast cancer accounts for up to 75% of all breast cancer cases [1]. Estrogen, predominantly in the form of estradiol (E_2), binds to ER to drive tumor cell growth through up-regulation of genes that support mitosis and cell cycle progression [2]. Tamoxifen and raloxifene inhibit breast tumor growth primarily by antagonizing E_2 whereas aromatase inhibitors, given post-menopause, act by blocking peripheral and intratumoral E_2 synthesis [3]. Tamoxifen is also used as a chemopreventive agent in women at risk for breast cancer [4]. In ER + breast cancer, a 5-year adjuvant treatment with tamoxifen given to inhibit residual tumor growth decreases recurrence by about 50 percent [5]. Breast tumors however can become growth adapted to attenuated E_2 signaling through dysregulated alternative signaling pathways [6]. Intrinsic or acquired resistance derives from subpopulations of cells within the tumor with an aggressive growth phenotype and migratory capacity [7] ultimately leading to tumor recurrence, drug resistance and metastasis [8]. In up to 90 percent of the cases however, when the primary tumor is ER+, lymph node and distant metastases retain ER expression even though their growth may have escaped hormonal control [9]. Notably, about 15% of invasive

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http://dx.doi.org/10.1016/j.bbrc.2015.01.004
0006-291X/

and ER + breast tumors also overexpress HER2 [10] which may confer estrogen-independent growth; in those cases, the role of estrogen signaling in tumor biology is unclear.

It is well known that in ER + breast cancer cells, E_2 also inhibits invasiveness and tumor progression, *in vitro* and *in vivo*, whether or not their growth is hormone-sensitive [11–17]. This is believed to be the reason that primary luminal breast tumors are less aggressive than basal-like tumors. Indeed, recent studies have revealed that, in postmenopausal women with prior hysterectomy, hormone replacement with estrogen as a monotherapy is actually associated with a persistent decrease in the frequency of invasive breast cancer [18]. Understanding mechanisms underlying the effects of E_2 and anti-estrogens on breast tumor cell invasiveness is potentially highly significant in addressing the problem of tumor progression within subpopulations of cells, such as ER+/HER2+ cells that are resistant to anti-estrogens. This is because in ER + tumors, anti-estrogen treatment could produce the undesirable collateral effect of suppressing the ability of E_2 to inhibit invasiveness in the resistant (e.g., ER+/HER2+) cells. This is a particularly significant issue because breast tumor cells could migrate at a relatively early stage from the primary tumor to distal sites [7].

The ERBB2 gene product, HER2 supports invasive and metastatic properties in several types of cancer and, in breast cancer, its amplification enables tumors to grow independent of estrogen. In one study, ectopic overexpression of HER2 in the ER-negative MDA-MB-435 cells enhanced their invasiveness [19]. In a second study, ectopic overexpression of HER2 increased brain metastasis in the ER-negative MDA-MD-231 cells [20]. Brain metastases of breast tumors are enriched for HER2 expression [20]. As the ERBB2 gene is known to be repressed by E_2 in ER + breast cancer cells [21], it has been suggested that down-regulation of HER2 by E_2 might contribute to the relatively low invasiveness of luminal breast tumors [14]. However, in this case, a causal link to HER2 down-regulation has not been demonstrated. Indeed, although HER2 amplification supports E_2-independent growth in ER + breast cancer cells and a basal level of HER2 may support invasiveness of ER + cells, a role for overexpression of endogenous HER2 in invasiveness has not been established in ER + breast cancer cells [22].

When bound to E_2, ER could either transactivate or transrepress its direct target genes. In either case, this could directly or indirectly result in both up-regulation and down-regulation of a host of genes that have diverse roles in breast tumor growth and progression. The mode of direct interaction of agonist bound ER with target genes that it transrepresses is fundamentally different from its interaction with genes that it transactivates, as gene repression involves agonist-induced recruitment of co-repressors rather than co-activators [23,24]. The mechanistic difference between transactivation vs. transrepression by E_2-bound ER offers an opportunity for mechanism-based targeting of E_2 action in a more functionally selective manner. The E_2-induced and co-activator dependent transactivation by ER is known to be necessary for E_2-dependent tumor cell growth. However, the relative importance of E_2-dependent transactivation vs. transrepression by ER in the ability of E_2 to regulate invasiveness is unclear.

It was therefore undertaken to directly examine the role of E_2-dependent transactivation vs. transrepression by ER in regulation of invasiveness by E_2 in ER+/HER2+ breast cancer cells.

2. Materials and methods

2.1. Chemicals and reagents

Cell culture media, charcoal-stripped fetal bovine serum, reagents for real time PCR, primers and TaqMan probes were from Life Technologies (Carlsbad, CA). 17β-estradiol (E_2) and 4-hydroxytamoxifen (4-OHT) were from Sigma Aldrich (St. Louis, MO). Phenol red-free growth factor reduced Matrigel (#356231) and Calcein AM Fluorescent Dye (#354216) were from BD Biosciences (San Jose, CA). Affinity purified anti-human ER (sc-543), anti-human Neu (sc-33684) and anti-human GAPDH antibodies (sc-44724) were from Santa Cruz Biotechnologies (Santa Cruz, CA).

2.2. siRNA

The small interfering RNAs (siRNA) for HER2 (#J-003126-20), ESR1 (#J-003401-12), CREBBP (#J-003477-08), EP300 (#J-003486-11) and Non-targeting siRNA (#D-001810-02) were from Thermo Fisher Scientific (Waltham, MA).

2.3. Cell culture and hormone depletion

BT474 breast cancer cells (American type Culture Collection) were cultured in DMEM supplemented with FBS (10%), penicillin (100unit/ml), streptomycin (100 μg/ml) and L-glutamine (2 mM). For hormone depletion, the cells were cultured in phenol red-free DMEM medium supplemented with 10% charcoal-stripped FBS (v/v) and incubated at 37°C with 5% CO_2 for 48 h–72 h prior to treatments

2.4. Transfection

Hormone depleted cells were plated to 20% confluence in phenol red-free DMEM medium supplemented with 10% charcoal-stripped FBS and transfected with the appropriate siRNA using Dharmafect 1 (Thermo Fisher Scientific, Waltham, MA) according to the vendor's protocol.

2.5. Mutagenesis

The ER-L372R mutant was generated by creating a point mutation in ERα cDNA at nucleotide position 1116 by converting the codon CTC to CGC using the QuickChange II site-Directed Mutagenesis kit (Agilent Technologies, Santa Clara, CA). The wild type ER cDNA and ER-L372R mutant cDNA were inserted in the pCDH lentiviral vector at the SalI site in the polylinker.

2.6. Lentiviral transduction

Lentiviral particles containing the appropriate pCDH plasmid were generated as described [25]. 48 h before transduction, BT474 cells were plated in phenol red-free DMEM medium with 10% heat inactivated charcoal-stripped FBS at 25% confluence. Cells were transduced with either pCDH-ER lentivirus or pCDH-ER-L372R lentivirus with polybrene (8 μg/mL) for 5 h followed by a similar second lentiviral transduction for an additional 5 h. After the second transduction, the virus was replaced with fresh phenol red-free medium containing 10% charcoal-stripped FBS.

2.7. Western blot analysis

Western blots were carried out as previously described [25].

2.8. RNA isolation, reverse transcription PCR and real time PCR

Total RNA was isolated and reverse transcribed as described [25]. cDNA was measured by quantitative real time PCR using the StepOne Plus Real time PCR System (Life Technologies, Carlsbad, CA). All of the mRNA measurements were carried out using biological triplicate samples and C_T values were normalized to those of GAPDH.

2.9. Cell proliferation assay

BT474 cells were seeded in 96-well plates at 20% confluence (1×10^4 cells/well) in phenol-red free media supplemented with 10% charcoal-stripped FBS (v/v) and incubated at 37 °C with 5% CO_2. The cells were treated with vehicle (ethanol) or E_2 (1 nM). Cell viability was determined using the MTT assay, as previously described [25]

2.10. Transwell invasion assay

1×10^5 cells were suspended in serum-free/phenol red-free DMEM medium and added in the top chamber of fluoroblok cell culture inserts (#351152; 8 μM pore membrane; BD Biosciences, Bedford, MA) coated with matrigel (0.2 mg/mL). 20% FBS in phenol-red free DMEM media was used as the chemoattractant. The appropriate treatments were included in both the top and the bottom chambers. Cells were allowed to invade for 24 h. Invaded cells on the bottom surface of the insert were labeled with Calcein AM (2 μg/ml - 4 μg/ml) for 1 h at 37 °C in the dark and imaged using a fluorescent microscope. The invasion assay for each treatment was performed in triplicate wells. Images were captured from each well from five fields of view using a 4× objective. The images were processed using the Image J software [26] and the number of cells invaded was quantified on the basis of brightness and pixel size.

2.11. Statistical analyses

Experimental values are presented as mean ± s.d. The statistical significance of differences (*P* value) between values being compared was determined using ANOVA. The *P* values are noted in the figures.

3. Results

3.1. Estrogen suppresses invasiveness in HER2-amplified ER + cells and this is antagonized by tamoxifen

We first confirmed that under our experimental conditions, we were able to reproduce the ability of E_2 to suppress invasiveness in a tamoxifen-sensitive manner in HER2-amplified ER + breast cancer cells using the well-established BT474 (ER+/PR+/HER2+) cell line model. E_2 strongly inhibited invasiveness in these cells and tamoxifen blocked this effect of E_2 (Fig. 1A).

3.2. Inhibition of invasiveness by estrogen is dependent on ER in ER+/HER2+ breast cancer cells

Certain cellular effects of E_2 could be mediated by membrane receptors rather than ER. In BT474 cells, depletion of ER (Fig. 1B) prevented E_2 mediated activation of its classical target genes (Fig. 1C). The ability of E_2 to inhibit invasiveness was also abrogated by knocking down ER (Fig. 1D) confirming ER-dependence of this effect. Knocking down ER also increased the basal level of invasiveness (Fig. 1D), indicating an additional hormone-independent effect of ER on invasiveness, as observed when ER-negative cells were transfected with ectopic ER [12].

3.3. A co-repressor binding site mutant of ER disrupts the ability of estrogen to inhibit invasiveness in ER+/HER2+ breast cancer cells

There is considerable functional redundancy or overlap among ER co-repressors. Therefore the best approach to specifically interrogating the role of E_2-dependent transrepression by ER in regulation of invasiveness by E_2 is to test an ER mutant incapable of co-repressor binding rather than knocking down a co-repressor. Mutation of L372 in ER to R372 is known to attenuate the ability of ER to interact with the CoRNR box motifs of ER co-repressors without affecting interaction with the LXXLL motifs of ER co-activators [27]. We overexpressed wt ER and the ER L372R mutant in BT474 cells using lentiviral transduction to achieve a 6–8 fold increase in the expression of ER mRNAs, accompanied by increases in protein expression (Fig. 2A). The ectopic mutant ER showed the expected dominant-negative effect on gene repression by E_2 on its direct target genes, IGFBP3 and S100P (Fig. 2B) without affecting activation of its direct target genes, PGR, GREB1 and pS2 (Fig. 2C). In contrast, the control ectopic wt ER retained both the gene repression (Fig. 2B) and the gene activation (Fig. 2C) by E_2. The transduced mutant ER but not wt ER abrogated inhibition of invasiveness by E_2 (Fig. 2D). The results demonstrate a causal link between transrepression by E_2 and the role of the hormone in suppression of invasiveness.

3.4. Attenuation of estrogen-dependent transactivation by ER does not affect regulation of invasiveness by estrogen in ER+/HER2+ breast cancer cells

The general ER co-activators, CBP and p300 together support the ability of E_2 to activate most of its direct target genes. In BT474 cells, a partial combined knockdown of CBP and p300 (Fig. 3A) was adequate to virtually completely block transactivation by E_2 (Fig. 3B) without affecting transrepression (Fig. 3C) of its direct target genes. The combined knockdown of CBP and p300 did not affect the ability of E_2 to inhibit invasiveness of BT474 cells (Fig. 3D). These results demonstrate that in contrast to the role of E_2-dependent transrepression noted above, E_2-dependent transactivation by ER is not required for regulation of invasiveness by E_2 in ER+/HER2+ breast cancer cells.

3.5. Suppression of invasiveness by estrogen is independent of regulation of HER2 in ER+/HER2+ breast cancer cells

Knocking down the overexpressed HER2 in BT474 cells to below its E_2-repressed levels (Fig. 4A) did not decrease invasiveness indicating that in these cells high levels of HER2 were not needed for their invasiveness (Fig. 4B). Moreover, knocking down HER2 to levels lower than the E_2-repressed levels failed to show any effect on the ability of E_2 to suppress invasiveness (Fig. 4B). On the other hand, the reduction in HER2 level did inhibit the growth of BT474 cells (Fig. 4C). The results demonstrate that HER2 does not play a role in the regulation of invasiveness by E_2 in ER+/HER2+ cells.

4. Discussion

The ability of anti-estrogens to oppose regulation of invasiveness of breast tumor cells by E_2 may be expected to negatively impact the outcome of anti-estrogen adjuvant therapy when the treated tumor contains ER + cells that have intrinsic or acquired resistance to the therapy. As HER2 amplification represents a well-established mechanism of resistance of ER + breast cancer cells to anti-estrogen therapy, we used the ER+/HER2+ BT474 cell line as a model for this study. It is well known that E_2-dependent transactivation by ER is critical for the ability of E_2 to support the growth of hormone-dependent breast cancer cells as demonstrated by the need for ER coactivators to support tumor growth [28,29]. In this study of ER+/HER2+ breast cancer cells, knocking down the general ER coactivators CBP and p300 did prevent transactivation by E_2/ER but did not affect the ability of E_2 to suppress invasiveness. Further, a causative link between the agonist-dependent

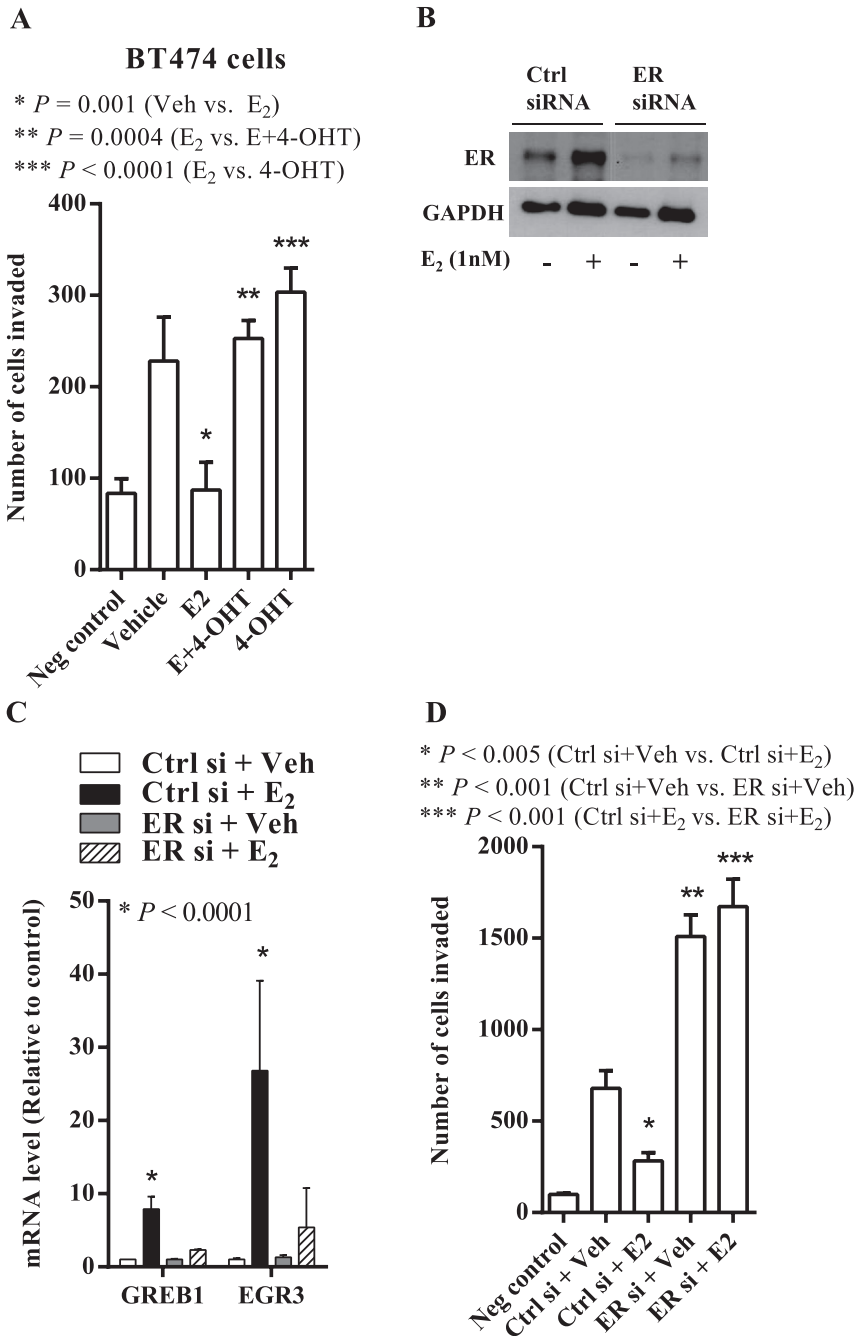

Fig. 1. Effect of E_2 and 4-OHT on invasiveness of BT474 cells and the role of ER. Hormone depleted BT474 cells were treated with either vehicle (ethanol), E_2 (1 nM), 4-OHT (500 nM) or E_2 (1 nM) plus 4-OHT (500 nM) for 72 h. Cells were subjected to the transwell invasion assay with the appropriate treatments (vehicle, E_2, 4-OHT or E_2 plus 4-OHT) included in both the top and the bottom chambers; FBS (chemoattractant) was excluded in the negative control (*Panel A*). Hormone depleted BT474 cells were transfected with control siRNA or ER siRNA. 24 h after transfection, cells were treated with either vehicle (ethanol) or E_2 (1 nM) for 48 h. Whole cell lysates were extracted to measure ER levels by western blot using GAPDH as the loading control (*Panel B*) and RNA was extracted to measure mRNA levels for the indicated genes by real time RT-PCR (*Panel C*) or cells were trypsinized and subjected to the transwell invasion assay (*Panel D*). In *Panel D*, FBS (chemoattractant) was excluded in the negative control.

transrepression mechanism of ER and the inhibition of invasiveness by E_2 was established in the ER+/HER2+ cells by taking advantage of the co-repressor dependence of ER for repression of its direct target genes. A mutation in the co-repressor binding site of ER abrogated both the gene repression and inhibition of invasiveness by E_2 but not E_2-dependent transactivation. The results indicate that the ability of E_2 to induce transrepression by ER is critical for suppression of invasiveness by the hormone rather than its ability to induce transactivation.

E_2 is known to partially repress the ERRB2 gene in ER + breast cancer cells [21]. By testing the effect of reducing endogenous HER2 to a level below the E_2-regulated level in BT474 cells, this study demonstrated that the partial downregulation of HER2 by E_2 could not account for the ability of E_2 to regulate invasiveness and that this must occur through other E_2 target genes.

The mode of E_2-dependent transrepression by ER is fundamentally different from that of transactivation by ER in that it involves agonist-induced co-repressor recruitment by ER, but there

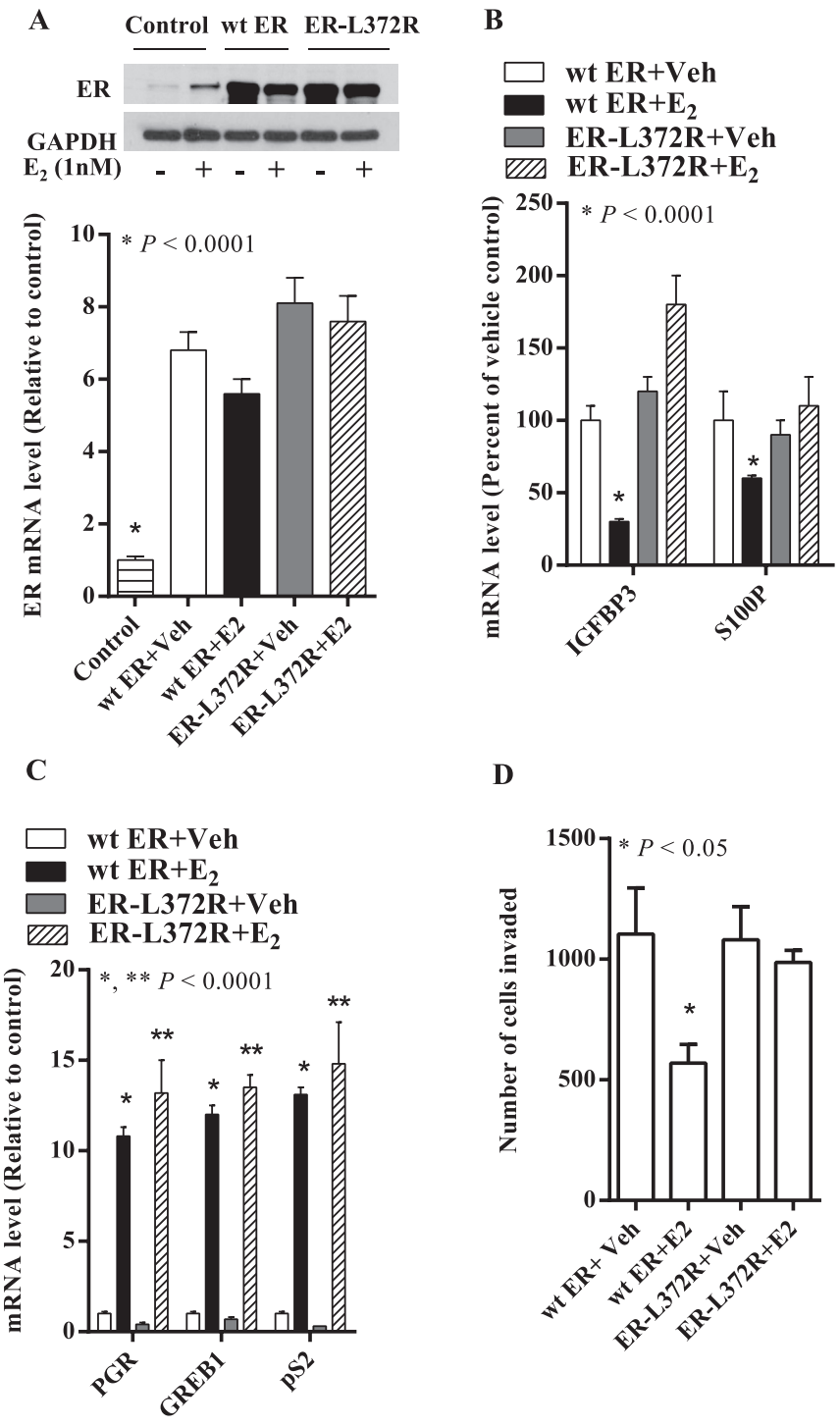

Fig. 2. Ectopic overexpression of wild-type ER or ER (L372R) in BT474 cells and the effect on regulation of gene expression and invasiveness by E_2. BT474 cells were plated in media containing heat-inactivated charcoal-stripped FBS 48 h prior to lentiviral transduction with wt ER or ER (L372R). 72 h after transduction, cells were treated with either vehicle (ethanol) or E_2 (1 nM). After 48 h of treatment, cells were harvested for RNA extraction and the mRNA expression levels of ER (*Panel A*) and other indicated genes (*Panels B and C*) were measured using real time RT-PCR. Whole cell lysates were also extracted to measure ER levels by western blot using GAPDH as the loading control (*Panel A, inset*). Cells were also trypsinized and subjected to the transwell invasion assay (*Panel D*).

could also be additional mechanistic differences. We have previously demonstrated a mechanistically distinct aspect of gene repression by E_2 wherein, the hormone caused ER and co-repressor recruitment to the target folate receptor α (FRα) gene by TAFII30 [23]. In contrast to the classical mechanism of tamoxifen action, tamoxifen dissociated ER from the FRα gene; moreover, placing a classical estrogen response element (ERE) upstream of the FRα gene promoter caused a switch to gene activation by E_2 [23]. The studies suggested possible mechanistic differences between gene repression and the classical model of gene activation, both with respect to E_2 action and the manner in which tamoxifen antagonized it. This view is also supported by the observation from global chromatin

binding studies that, in contrast to E_2 activated genes, E_2 repressed genes are not enriched for associated canonical EREs [30]. Mechanistic differences between transactivation and transrepression by E_2-bound ER could potentially be exploited to develop agents that would selectively antagonize this transactivation component of E_2 action.

In conclusion, this study provides new insights into possible mechanisms contributing to emergence of more invasive ER + tumor cells in the course of anti-estrogen therapy. The ability of anti-estrogens to antagonize E_2 induced transactivation of its direct target genes by ER is known to be critical for their anti-tumor effect; however, we conclude that in the anti-estrogen resistant ER+/HER2+ breast cancer cells, hormone-induced transactivation by ER does not impact the ability of E_2 to regulate invasiveness. On the other hand, in ER+/HER2+ breast cancer cells, E_2-induced transrepression by ER is critical for suppression of invasiveness by the hormone and therefore antagonism of this function of ER would encourage an invasive phenotype. Therefore, a superior treatment

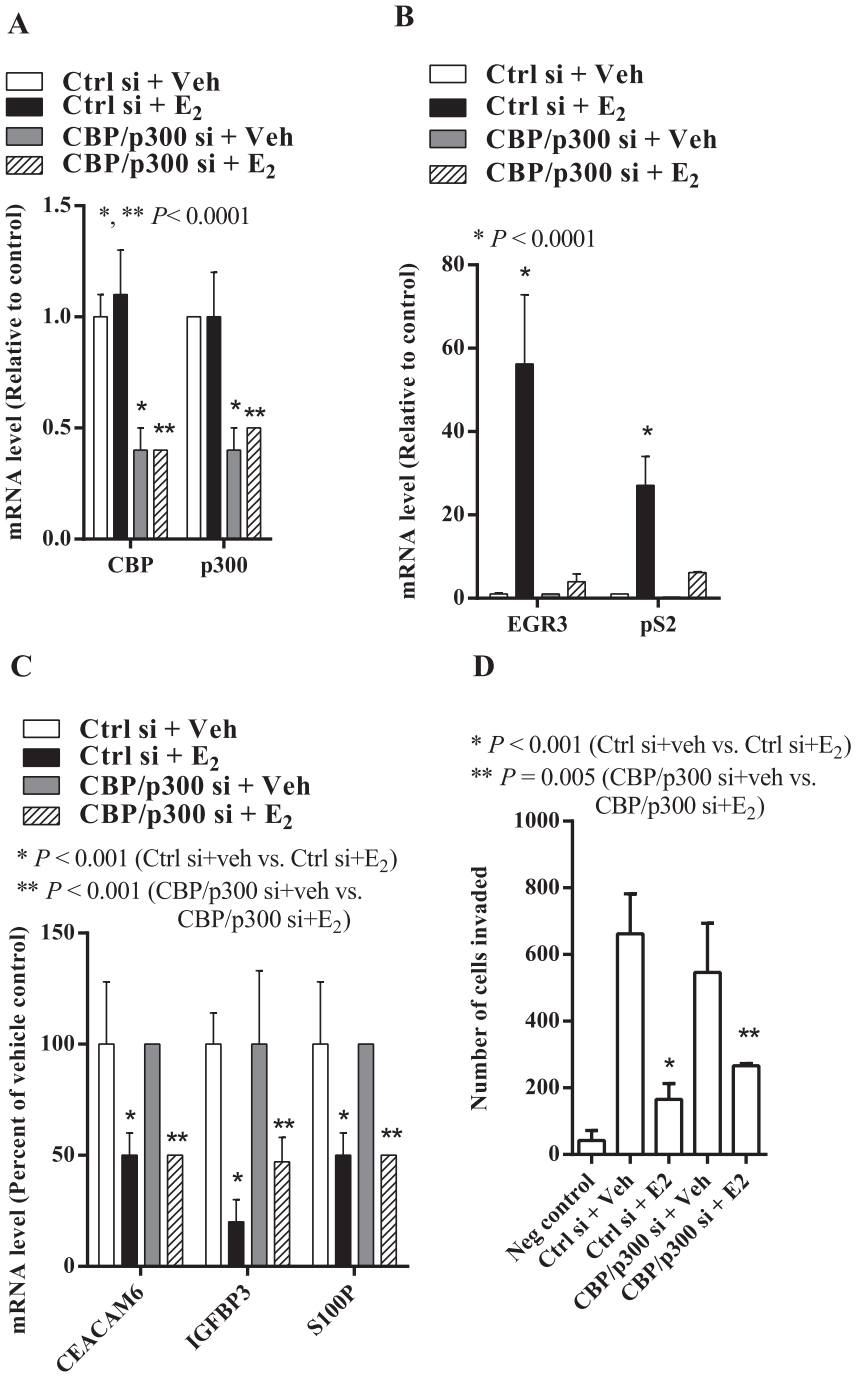

Fig. 3. Effect of knocking down CBP and p300 on gene regulation by E_2 and regulation of invasiveness by E_2. Hormone depleted BT474 cells were transfected with control siRNA or CBP and p300 siRNA. 24 h after transfection, cells were treated with either vehicle (ethanol) or E_2 (1 nM). RNA was extracted after 48 h of treatment to measure mRNA levels for the indicated genes by real time RT-PCR (*Panels A-C*). Cells were also trypsinized and subjected to the transwell invasion assay; FBS (chemoattractant) was excluded in the negative control (*Panel D*).

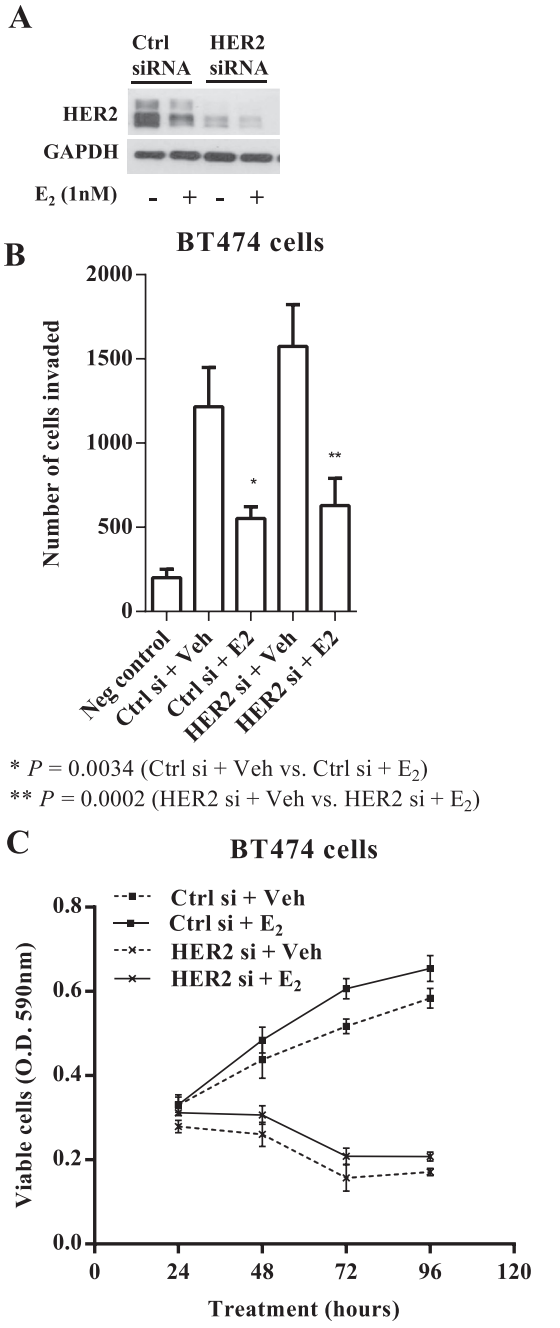

Fig. 4. Effect of knocking down HER2 on growth and regulation of invasiveness by E_2. Hormone depleted BT474 cells transfected with either control siRNA or HER2 siRNA. 24 h after transfection, cells were treated with either vehicle (ethanol) or E_2 (1 nM). After 48 h of treatment, whole cell lysates were extracted to measure HER2 levels by western blot using GAPDH as the loading control (*Panel A*). Cells were also trypsinized and subjected to the transwell invasion assay; FBS (chemoattractant) was excluded in the negative control (*Panel B*). 24 h after transfection, cells were trypsinized and seeded in 96-well plates in six replicates. 24 h later, cells were treated with vehicle (ethanol) or E_2 (1 nM) for the indicated periods and cell viability was measured by the MTT assay (*Panel C*).

for ER + breast cancer may require drugs that would selectively inhibit the hormone-dependent transactivation function of ER without disrupting transrepression of direct E_2 target genes. The agents could include novel selective ER modulators or selective inhibitors of the binding of certain co-activators to ER. Such drugs would inhibit E_2-dependent tumor growth without compromising suppression of invasiveness by E_2.

Conflict of interest

None.

Acknowledgments

This work was supported by NIH grant 5R01CA140690 and The Harold & Helen McMaster endowment to M.R. Yanfang Huang provided technical assistance.

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