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Reciprocal regulation of ZEB1 and AR in triple negative breast cancer cells

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Abstract

Zinc-finger enhancer binding protein (ZEB1) is a transcription factor involved in the progression of cancer primarily through promoting epithelial to mesenchymal transition (EMT). ZEB1 represses the expression of E-cadherin by binding to E-box sequences in the promoter, thus

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decreasing epithelial differentiation. We show that ZEB1 and androgen receptor (AR) cross-talk in triple negative breast cancer cell lines. Chromatin immunoprecipitation analysis demonstrates that ZEB1 binds directly to the E-box located in the AR promoter. ZEB1 suppression by stably transfecting shRNA in a triple negative breast cancer cell line resulted in a decrease of AR mRNA, protein, and AR downstream targets. ZEB1 knockdown in triple negative breast cancer cells sensitized the cells to bicalutamide by reducing migration compared to the control cells. Conversely, blockade of AR signaling with bicalutamide resulted in a suppression of ZEB1 protein expression in two triple negative breast cancer cell lines. Furthermore, using a breast cancer tissue microarray, a majority of triple negative breast cancers exhibit positive staining for both ZEB1 and AR. Taken together, these results indicate that ZEB1 and AR regulate each other to promote cell migration in triple negative breast cancer cells.

Keywords

ZEB1; AR; Breast cancer; Triple negative

Introduction

Hormones play a pivotal role in endocrine-mediated tumorigenesis and influence cancer cell growth and progression [1]. The transition to hormone insensitivity remains a major challenge in the management of breast cancer (BC). Anti-estrogen treatment is a mainstay in BC therapy for patients with early stage tumors; however, a subset of patients develop BC that can grow independent of estrogens [2, 3]. Estrogen receptor (ER) negative, progesterone receptor (PR) negative, HER2 negative (triple negative) BC represent approximately 25–30% of all BC and generally has a more aggressive clinical course [4]. There is a need for new therapies to treat triple negative cancers due to the lack of effective targeted treatments available for this subset of breast cancer. A significant association has been found between the expression of androgen receptor (AR), ER, and PR as determined by immunohistochemistry [5]. AR is expressed in 60–70% of BC and implicated in BC biology [5–7]. Similar to other steroid hormone receptors, AR is a ligand-activated transcription factor that modulates diverse biological effects through regulation of downstream target genes [7]. Upon activation by ligand, the AR translocates to the nucleus, where it binds to the androgen response element of target genes and recruits other cofactors to regulate transcription [7, 8].

The heterogeneity of AR expression in BC and the complexity of AR signaling results in variability in the effects of androgens on BC proliferation [9]. Although the functional role of androgens in BC has yet to be clearly delineated, several BC cell lines are growth inhibited by the addition of androgens [7, 9]. However, many are growth stimulated and may be androgen dependent [9, 10]. Androgens are biologically significant in vivo. Animal models implicate androgen signaling in breast carcinoma progression [11]. Wong et al. demonstrated that testosterone in combination with estrogen induced a high incidence of mammary carcinoma in rats and treatment with the antiandrogen flutamide, produced significantly smaller tumors compared to the controls [12].

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Approximately 60–70% of breast tumors are positive for AR [6]. Some studies have demonstrated that a significant percentage of tumors are positive for AR and negative for ER and PR [5, 13]. This suggests that AR may function independent of ER and PR to cross-talk with other factors to enhance tumor progression. ZEB1 (zinc-finger enhancer binding protein; ZFHX1A, AREB6, or δEF1) is a transcription factor that plays a key role in cancer progression by regulating the epithelial to mesenchymal transition (EMT) in breast, prostate, ovarian and colorectal cancers [14–19]. ZEB1 can act as a transcriptional repressor or activator [20]; ZEB1 induces EMT primarily through repressing E-cadherin, which causes cells to lose their polarity and convert to a mesenchymal phenotype, leading to increased tumor migration and invasion both in vitro and in vivo [21, 22]. The transcriptional activation of ZEB1 has also been documented; ZEB1 contains several structural elements shown to be the active domains of other transcriptional activators [23, 24].

ZEB1 expressing tumor cells exhibit impaired adhesion and invaded host tissues more readily [25]. Likewise, in invasive ductal carcinomas of the breast, ZEB1 upregulation correlated with epithelial dedifferentiation and is highly expressed in tumor-associated stroma cells [25]. Various growth factors such as IGF-I and EGF regulate ZEB1 expression in cancer cells [19, 26]. In addition to growth factors, previous reports showed that hormones regulate ZEB1 expression. Estradiol and progesterone upregulated ZEB1 in the endometrial stroma and myometrium of the mouse and human uterus [22]. In the present study, we investigated whether ZEB1 plays a role in crosstalk with androgen signaling in triple negative BC cells, which is thereby involved in androgen regulation of BC cell growth. We demonstrate that inhibition of ZEB1 in these cells not only results in morphologic changes, but also results in a dramatic reduction in AR mRNA and protein and sensitizes the cells to anti-androgens. Conversely, inhibiting AR signaling with bicalutamide suppressed ZEB1 expression. Thus, ZEB1 cross-talk with AR signaling appears to be a critical pathway in metastatic BC cells.

Materials and methods

Cell lines and culture

MDA-MB-231, T4-7D, BT-549, and MCF-7 cells were purchased from ATCC (Manassas, VA). MDA-MB-435 cells were generously donated by Dr. Lily Yang (Emory University). The cell lines were cultured in RPMI-1640 (Invitrogen, Carlsbad, CA) supplemented with 5% FBS.

Antibodies and reagents

The rabbit ZEB1 (H-102), mouse androgen receptor (441), and goat PSA antibodies were purchased from Santa Cruz biotechnology (Santa Cruz, CA). The mouse monoclonal FKBP51 and mouse monoclonal E-cadherin were purchased from BD Transduction laboratories (San Diego, CA). The synthetic androgen R1881 was purchased from Perkin Elmer (Boston, MA), dihydrotestosterone (DHT) was purchased from Sigma–aldrich (St. Louis, MO), bicalutamide was obtained from Astrazenaca (Wilmington, DE). Puromycin was purchased from Calbiochem (Darmstadt, Germany).

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RNA preparation and semiquantitative RT–PCR

Cells were grown to reach 70–80% confluence and total RNA was harvested using Qiagen RNeasy RNA extraction kit (Qiagen, Hilden, Germany). cDNA was synthesized from MDA-MB-231-GAPDH shRNA and MDA-MB-231-ZEB1shRNAG_{11} cells using the Superscript first strand cDNA synthesis kit (Invitrogen, Carlsbad, CA). cDNAs were used for PCR analysis using the following oligonucleotide primers specific for ZEB1: forward (5′-TTCAGCATCACCAGGCAGTC-3′) and reverse (5′-GAGTGGAG GAGGCTGAGTAG-3′), the PCR conditions were as follows: 40 cycles at 94°C for 30 s, annealed at 53°C for 30 s, extended at 72°C for 2 min and a final extension at 72°C for 7 min. 28SrRNA: forward (5′-ACGGTAACGCAGGTGTC CT-3′) and reverse (5′-CCTCTCGTACTGAGCAGGA-3′), The PCR conditions were as follows: 29 cycles at 95°C for 40 s, annealed at 56°C for 30 s and extension for 1 min and a final extension at 72°C for 7 min. E-cadherin: forward (5′-TCCATTTCTTGGTCTACGCC-3′) and reverse (5′-CACCTTCAGCCAACCTGTTT-3′). The PCR conditions were as follows: 35 cycles at 95°C for 30 s, annealed at 60°C for 30 s and extension for 1 min at 72°C followed by a final extension for 8 min at 72°C. AR: (5′-ATGGCTGTCATTCAGTACTCCTGGA-3′) reverse (5′-AGATGGGCTTGACTTTCCCAGAAAG-3′), the PCR conditions were as follows: 35 cycles at 94°C for 1 min, annealed at 55°C for 1 min and extended 1 min at 72°C and a final extension at 72°C for 7 min. PCR reactions were run on a 2% agarose gel. Bands were visualized under UV illumination.

Immunoblot analysis

Cells were lysed in a modified RIPA buffer: 1 M Tris, 5 M NaCl, 1% Triton X, 1 mM sodium orthovanadate and protease inhibitor cocktail (Roche, Mannheim, Germany). The lysates were freeze-thawed three times followed by a spin at 13,000×*g* for 30 min at 4°C. Whole cell lysates were quantitated using BCA protein assay reagents (Pierce, Rockford, IL). 30 μg of total protein were separated by SDS–polyacrylamide gel electrophoresis (10%) and transferred onto a PVDF membrane (Biorad, Hercules, CA). The membranes were blocked in 5% non-fat dry milk diluted in TBST (0.2 mol/l NaCl, 10 mmol/l Tris, pH 7.4, 0.2% Tween-20) for 1 h at room temperature, then incubated with primary antibodies overnight at 4°C. The following day the membranes were washed with TBS containing 0.2% Tween 20, the membranes were subsequently incubated with horse-radish peroxidase labeled secondary antibodies for 1 h at room temperature followed by washing with TBST. The signal was detected by incubation with enhanced chemiluminescence (ECL) reagents (Amersham-Pharmacia Biotech, Piscataway, NJ) and exposed on HyBlot CL autoradiography film (Denville Scientific, Metuchen, NJ). In order to assess sample loading, the membrane was stripped with Restore Western Blot Stripping Buffer (Pierce, Rockford, IL) and probed with a β-actin antibody. Images were resized using Adobe Photoshop software followed by densitometric analysis using Image J software (http://rsb.info.nih.gov/ij/, Bethesda, MD).

Immunoprecipitation

Whole cell lysates (500 μg) from MDA-MB-231 and MDA-MB-435 were collected using RIPA lysis buffer. Following clearing of lysates, the specified antibody or IgG was added

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and reactions were incubated at 4°C overnight with rotation. IPs were washed three times with IP PBS-T (0.1% tween) at 4°C. For immunoblot analysis, loading buffer was added directly to the lysates and resolved on a 10% SDS–PAGE using standard procedures.

Chromatin immunoprecipitation (ChIP)

Chromatin immunoprecipitation analyses were done using a standard protocol [27]. Chromatin samples were sonicated on ice three times for 10 s each (i.e., until the average length of sheared genomic DNA was 600–800 bp) followed by centrifugation for 15 min. The immunoprecipitated DNA was ethanol precipitated and resuspended in 40 μl of H_2O. Initially, PCR was performed with different numbers of cycles and/or dilutions of input DNA to determine the linear range of amplification; all results shown fall within this range. Following 28–30 cycles of amplification, PCR products were run on 1% agarose gel and analyzed by ethidium bromide staining. The primers used for ChIP are as follows: AR promoter forward primer 5′-GCGTGGTTGCTCCCGCAAG-3′, AR promoter reverse primer 5′-GGGTAGACCCTTCCCAGCCC-3′.

Generation of stable MDA-MB-231 cells with ZEB1 knockdown

MDA-MB-231 ZEB1 knockdown stable cell lines were generated by short hairpin RNA (ZEB1-shRNAmir), shRNA targeted to GAPDH was used as a control. The four ZEB1-shRNAmir (B3, E5, G10, and G11) were purchased from Open Biosystems (Huntsville, AL) based on the GIPZ lentiviral vector. MDA-MB-231 cells were transfected with 10 μg of shRNA using lipofectamine 2000. After 48 h incubation, cells were selected for 8 weeks using puromycin (1 μg/ml) (Calbiochem).

Cell migration assay

Migration assays were performed using Boyden chambers containing polycarbonate inserts of 8 μm pore size membranes. The underside of the insert were coated with rat tail collagen 2.5 μg/cm^2 (BD biosciences). MDA-MB-231-GAPDH and MDA-MB-231-ZEB1-G_{11} (5×10^4) were seeded in quadruplicates in the top well of the inserts in phenol red free RPMI 1640 containing 5% charcoal-stripped FBS and treated with vehicle (ethanol), DHT (10 nM), bicalutamide (10 μM), or a combination of DHT and bicalutamide. After 6 h of incubation, cells that invaded through the collagen were fixed with formaldehyde, stained with 0.5% crystal violet, and counted to obtain relative migration. The average number of cells per field is represented.

Tissue microarrays and immunohistochemistry

The breast cancer tissue microarrays were purchased from Pantomics (San Francisco, CA). Antigen retrieval was done in 1× EDTA buffer (pH 8.0) using the LabVision PT-module. The immunohistochemistry assay was performed using DAKO LSAB 2 kit in a DAKO Autostainer (Dako-Cytomation, Glostrup, Denmark). The endogenous peroxidase was blocked with 3% hydrogen peroxide followed by incubation with primary ZEB1 (1:350 dilution) or AR antibody (Santa Cruz biotech) at a dilution of 1:100 for 1 h at room temperature. The tissues were then incubated with biotinylated secondary antibody (DakoCytomation, CA, USA) for 30 min followed by enzyme labeling with freshly prepared

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horseradish peroxidase-labeled streptavidin (DakoCytomation, CA, USA) at a dilution of 1:200 for 30 min. The developing chromogen DAB+ solution (DakoCytomation, CA, USA) was added for 5 min and then the sections were lightly counterstained 1:3 dilution with Hematoxylin in dH_2O (Richard-Allan Scientific, Kalamazoo, MI). The negative control consisted of non-immune mouse or rabbit IgG. The cores were scored by a pathologist as negative (0), weak (1), moderate (2), or strong (3) for ZEB1 and AR.

Statistical analysis

All data are representatives of three independent experiments with similar results. Statistical analysis was done using Microsoft Excel software. Significant differences were analyzed using Student's *t* test and two-tailed distribution. A *P* value of <0.005 was deemed significant. Error bars represent the standard deviation between triplicate experiments. The statistical significance between AR and ZEB1 protein expression in ER/PR+ and ER/PR– tissues was done using SPSS statistical software. A *P* value of <0.005 was deemed significant.

Results

Analysis of ZEB1 and AR in breast cancer cells and tissues

ZEB1 is involved in the malignant progression of prostate, lung, colorectal, and breast cancers [14, 15, 19, 25]. In order to correlate the expression of ZEB1 to AR, we evaluated their expression in a panel of ER/PR positive and ER/PR negative BC cells. AR was expressed in all the cell lines tested, but ZEB1 was expressed only in the ER/PR negative cell lines (BT-549, MDA-MB-435, and MDA-MB-231). In order to further investigate the relationship between ZEB1 and AR we conducted immunohistochemistry in a high-density tissue microarray. We found that a majority of malignant ER/PR negative breast cancer tissue (70% for ZEB1, 67% for AR) exhibited moderate or intense staining of AR and ZEB1 (Table 1). Furthermore, as shown in Fig. 1b, AR was present in both the cytoplasm and nucleus but, ZEB1 exhibited primarily cytoplasmic staining, with punctate nuclear staining in the tumor and nuclear staining in the stroma, as indicated by the arrows (Fig. 1b). Statistical analysis showed that both ZEB1 and AR positively correlate in ER/PR– breast cancer tissue ($P<0.001$).

ZEB1 suppression down-regulates AR mRNA and protein expression in vitro

In order to provide insight into the effect of ZEB1 on AR expression, we established stable ZEB1 knockdown in MDA-MB-231 cells, a metastatic breast cancer cell line in which it is highly expressed, using a lentivirus encoding ZEB1 shRNA or a lentivirus encoding against GAPDH. Downregulation of ZEB1 was evident in all four of the shRNA tested, in particular, ZEB1-shRNA-G_{11}, which exhibited an 8-fold decrease in ZEB1 transcripts and protein compared to the control transfected cells (Fig. 2a, b). As expected, suppression of ZEB1 restored E-cadherin protein and mRNA expression and concomitantly induced a switch in the morphology from mesenchymal to epithelial as indicated by the arrows (Fig. 2a–c). Interestingly, there was a marked decrease in AR, approximately 15-fold in ZEB1-shRNA-G_{11}, which, led to a decrease in PSA expression in these cells.

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ZEB1 activates gene transcription through direct interaction with the AR promoter

In order to examine whether ZEB1 can directly bind to the AR promoter, we analyzed the AR core promoter sequences and found a putative E-box, (CAGGTG), a binding sequences for ZEB1, located at nucleotide −117 to −123 of the AR gene (Fig. 3a). In order to demonstrate the binding of ZEB1 to the AR promoter, we performed a ChIP assay in MDA-MB-231 and MDA-MB-435 cells. MDA-MB-435 cells were chosen primarily because these cells like MDA-MB-231 cells are ER/PR/HER2 negative. In addition, MDA-MB-435 cells have comparable levels of ZEB1 and AR to MDA-MB-231 cells. In the chromatin fraction pulled down by an anti-ZEB1 antibody, we detected the AR promoter PCR fragments (Fig. 3b). However, AR promoter PCR fragments were not found in samples pulled down by a control IgG antibody. However, we were not able to find any putative AREs in the ZEB1 promoter, which, suggests that AR may regulate ZEB1 expression through an intermediary protein. By conducting an immunoprecipitation using the AR antibody, we determined that ZEB1 co-precipitated with AR. Conversely, AR pulled down with ZEB1 in both cell lines (Fig. 3c). Collectively, the results of the immunoprecipitations indicate that ZEB1 and AR interact in triple negative breast cancer cells.

Blockade of AR signaling leads to a decrease in ZEB1 expression

We next evaluated whether an AR antagonist, bicalutamide, could modulate ZEB1 expression. To this end, we carried out dose–response experiments in MDA-MB-435 and MDA-MB-231 treated with bicalutamide for 24 and 48 h, respectively, to determine the optimal concentration to reduce ZEB1 expression. Suppression of ZEB1 protein is evident at a 10 μM concentration in both cell lines (Fig. 4a). In order to confirm that AR signaling was blocked in these cells, we probed for FKBP51, an androgen regulated gene and determined that its expression is reduced with the highest dosage of bicalutamide. Conversely, treatment with dihydrotestosterone upregulates ZEB1 expression in the MDA-MB-231-ZEB1shRNAG_{11} cells (Fig. 4b).

Suppression of AR in ZEB1 knockdown cells decreases cell migration

Based of the above findings, we evaluated whether ZEB1-mediated down-regulation of AR in MDA-MB-231 cells result in decreased migration. As shown in Fig. 5a, exogenous DHT increased cell migration of control-shRNA cells by 4-fold. DHT had a more pronounced effect on the control-shRNA transfected cells because knockdown of ZEB1 reduces the expression of AR, thus lowering the amount of available receptor for the ligand to bind. Treatment with the AR inhibitor efficiently reduced the migration of both cell lines through the collagen coated inserts by up to 2.5-fold, $P<0.005$. More importantly, treatment with the anti-androgen suppressed the cell migration of the MDA-MB-231-ZEB1shRNAG_{11} cells to a greater extent compared to the MDA-MB-231-GAPDH shRNA cells. These results indicate that exogenous androgens exert migratory effects on triple negative BC cells, which appears to be dependent on the expression of ZEB1, indicating that ZEB1 and AR exert migratory effects on BC cells (Fig. 5).

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Discussion

Androgen signaling has important implications in breast cancer. Almost all the AR-positive breast tumors are also positive for prostate-specific antigen (PSA) (98%) and for the gross cystic disease fluid protein-15 (GCDFP-15) (92%), two androgen regulated genes, suggesting that AR is functional in human BC [28]. Doane et al. report of a subset of ER/PR negative BC, characterized by a hormonally regulated gene expression signature which is AR-dependent and androgen-induced cell growth in culture [10]. In addition, the androgen antagonist, flutamide, has been used for the treatment of BC [29].

A significant association has been made for tumors that are positive for AR and negative for ER and PR [5]. This suggests that AR can function independently of ER and PR and work in concert with other signal transduction pathways to contribute to an aggressive phenotype. We demonstrate that ZEB1, a transcription factor involved in EMT, and AR cross-talk to promote BC progression. It has been reported that other steroid hormones induce the expression of ZEB1. Richer et al. showed by microarray analysis that ZEB1 was upregulated 3.6-fold in T47D breast carcinoma cells stimulated with progesterone [30]. By differential display, the cDNA for the chick homolog of ZEB1, δEF1, is regulated by estrogen; both the mRNA and protein levels increase in response to estrogen [31]. Spoelstra et al. identified both estrogens and progestins as inducers of ZEB1 in both the stroma and myometrium in vivo with 6 and 24 h of treatment [22].

ZEB1 expression has been well documented in poorly differentiated BC tissues and highly metastatic BC cell lines [21, 25, 32]. ZEB1 is linked to cancer progression primarily through the induction of EMT [14, 15, 19, 21]. ZEB1 exerts its cellular effects by binding to highly conserved consensus sequences (E-boxes) to repress the expression of target genes [15]. This transcriptional repressor also reduces the expression of basement membrane components [21].We and others have shown that ZEB1 knockdown in MDA-MB-231 cells result in a morphological switch of the cells from mesenchymal to epithelial [14, 19, 32]. Expression of ZEB1 associated with EMT and selective loss of basement membrane in invasive tumor regions is a strong predictor of poor patient survival and metastasis [14]. Although ZEB1 is primarily characterized as a transcriptional repressor, the mouse homolog δEF1 has been shown to activate genes as well. δEF1 cooperates with the transcription factor USF to activate ovalbumin gene expression [33]. In addition, δEF1 can activate the VDR gene and a Na,K-ATPase promoter construct [34, 35]. Our studies indicate that ZEB1 plays a role in AR regulation. Based on our chIP analysis, we show that ZEB1 can bind directly to the E-box sequence on the androgen receptor promoter, which led us to believe that this interaction occurs in the nucleus. Immunohistochemistry illustrate that both proteins are expressed almost exclusively in ER/PR– breast cancer tissues. Although the staining for ZEB1 is primarily in the cytoplasm, both proteins are expressed in the nucleus. Stable knockdown of ZEB1 in MDA-MB-231, a triple negative BC cell line, reduced AR expression at the mRNA and protein level by more than 50% as well its downstream target PSA. This effect appears to be exclusive to ER/PR negative cells since overexpression of ZEB1 in T47D cells suppresses the expression of AR instead of activating it (data not shown), suggesting that ER may interfere with the ability of ZEB1 to suppress AR. We confirmed that AR signaling is functional in triple negative cells because treatment with the

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AR antagonist bicalutamide suppressed the expression of the FKBP51, anARtarget gene. In addition, bicalutamide treatment reduced the expression of ZEB1. Anose et al. recently published results illustrating the regulation of ZEB1 mRNA by androgens in a prostate cancer cell line [36]. However, in contrast to our results, they found that treatment with the anti-androgen, flutamide exhibited stimulation of PSA and ZEB-1 mRNA levels [36]. The underlying mechanisms of these differential effects of anti-androgens on ZEB1 in mechanisms in breast and prostate cancer cells is unclear.

Our results indicate that the bi-directional regulation of ZEB1 and AR in BC cells has biological significance, illustrated in fig. 6. Inhibition of ZEB1 has biological significance for triple negative cells treated with bicalutamide since MDA-MB-231-ZEB1-shRNA-G11 cells had suppressed motility in response to the anti-androgen.

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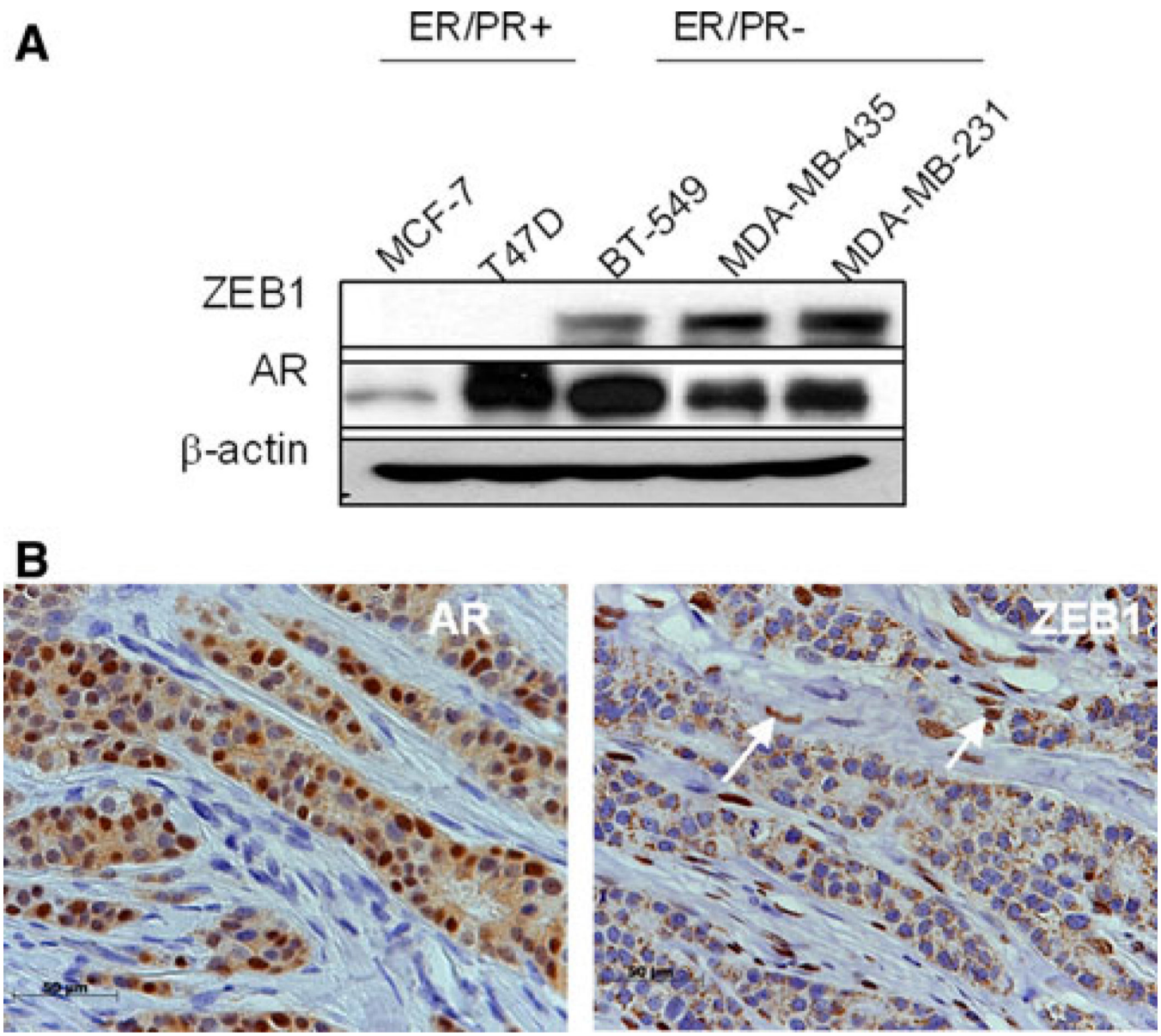

Fig. 1.

ZEB1 and androgen receptor expression in human breast cancer cells and tissues. **a** Western blot analysis of ZEB1 and AR in MCF-7, T47D, BT-549, MDA-MB-435, MDA-MB-231. **b** Representative images of ZEB1 and AR immunostaining in ER/PR negative breast cancer. Photos taken at 200×

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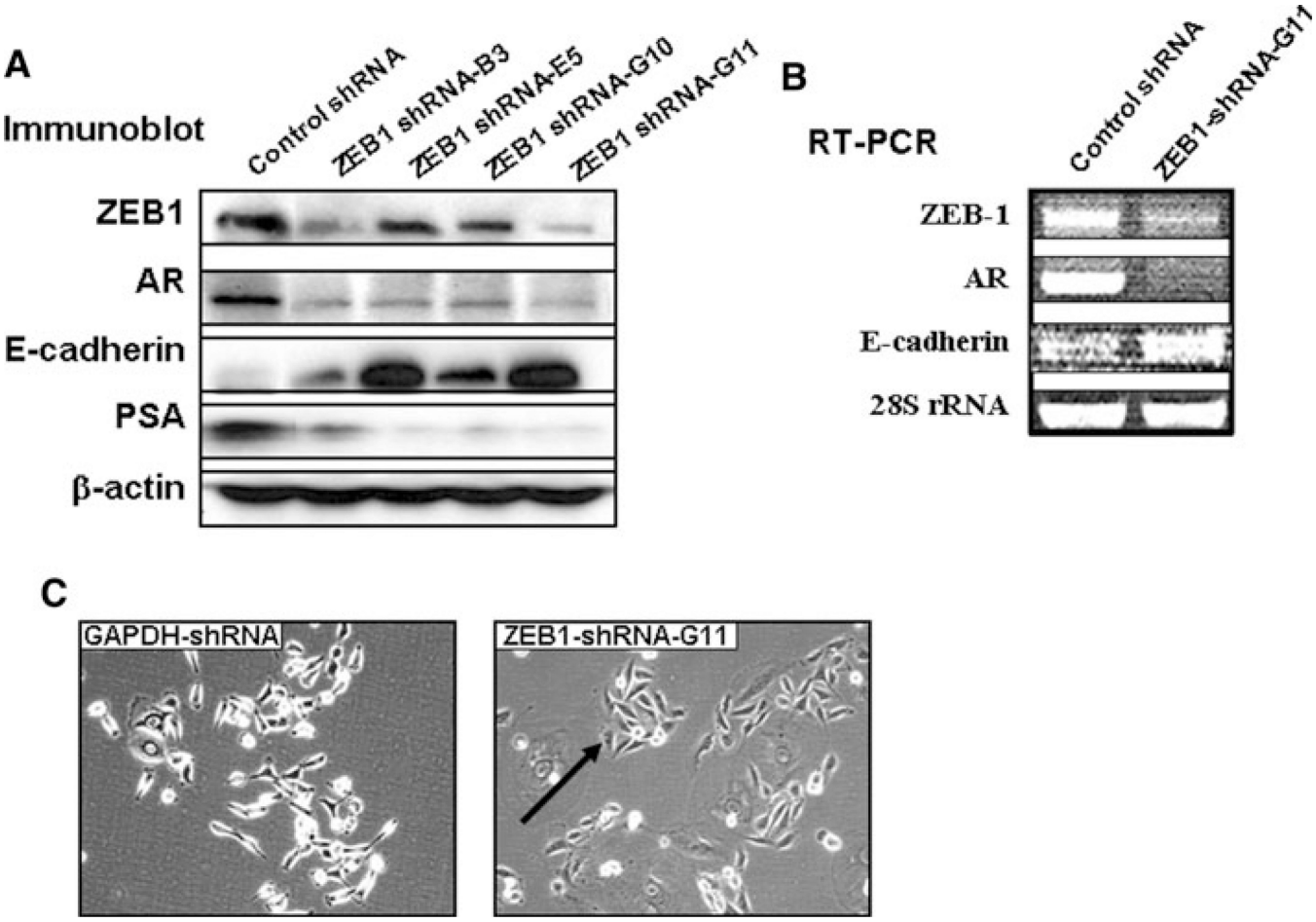

Fig. 2.

ZEB1 inhibition leads to decrease in AR expression in MDA-MB-231 cells. **a** Whole cell lysates of MDA-MB-231 cells stably transfected with ZEB1 shRNA were probed with the indicated antibodies. **b** Semiquantitative RT–PCR analysis of the ZEB1, AR, E-cadherin genes in the control and ZEB1 knockdown MDA-MB-231 cells. 28S rRNA RT–PCR was done to assess the quality of the cDNA and ensure equal amounts of cDNA used in the PCR reactions and β-actin was used as a loading control for the whole cell lysates. **c** MDA-MB-231 ZEB1-shRNAG_{11} exhibited an epithelial morphology compared to the control transfected cells. Photos taken at 100× magnification

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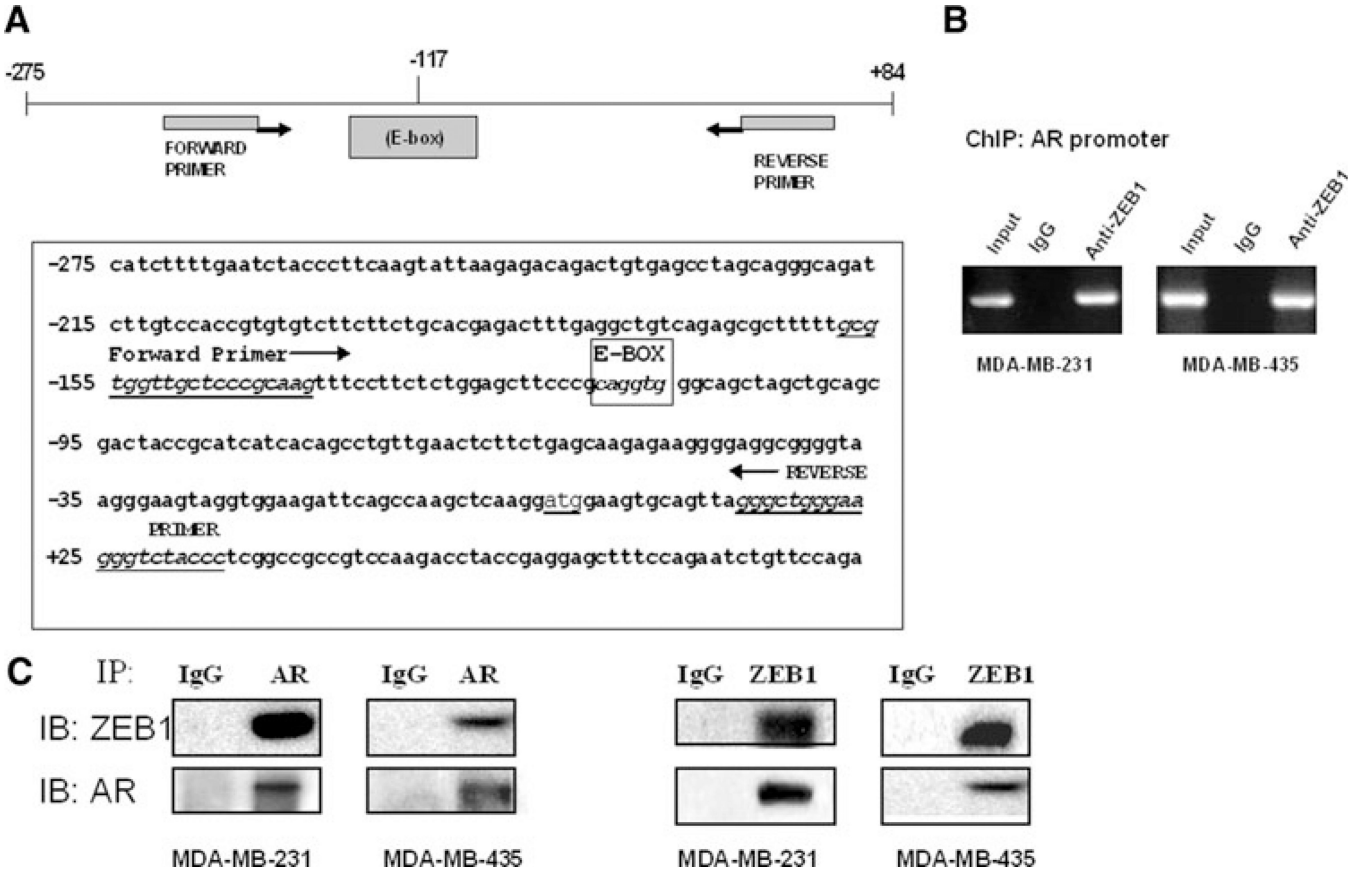

Fig. 3.

AR and ZEB1 physically interact in ER/PR negative breast cancer cells. **a** Schematic of potential E-box located in the AR promoter. **b** ZEB1 associates with the AR promoter at the chromatin level in breast cancer cells as shown by ChIP analysis. Rabbit ZEB1 antibody or Rabbit IgG (Control) were used for ChIP experiments. **c** ZEB1 was immunoprecipitated with AR antibody from cell lysates from MDA-MB-231 and MDA-MB-435 cells, conversely AR was immunoprecipitated with the ZEB1 antibody. Normal rabbit IgG as a control and immunoblotted

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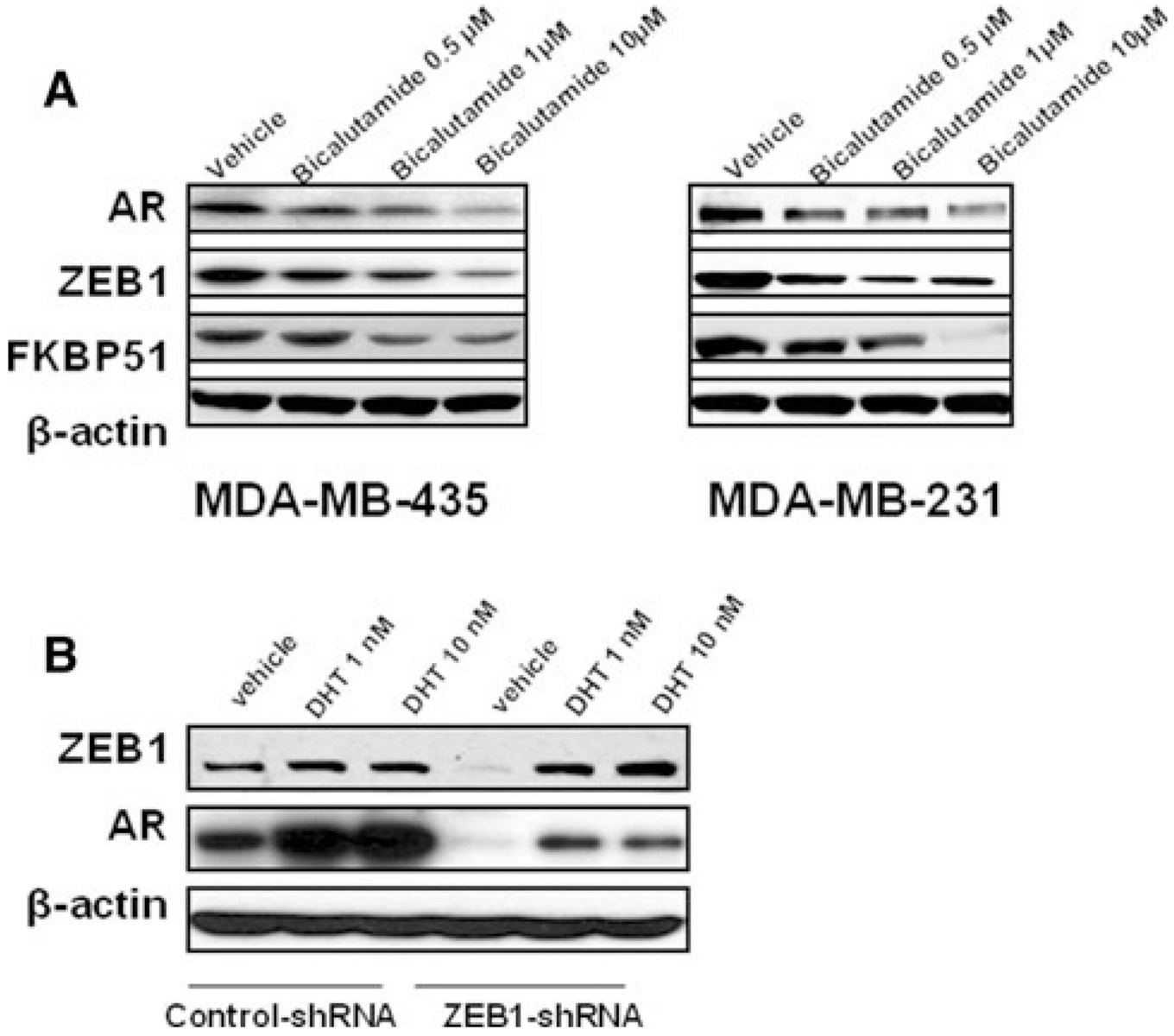

Fig. 4.

ZEB1 expression is modulated by AR signaling. **a** Dose– response experiments were carried out by incubating MDA-MB-435 and MDA-MB-231 cells cultured in phenol red free RPMI, supplemented with 5% charcoal-stripped FBS with the indicated doses of bicalutamide for 24 and 48 h. The whole cell lysates were probed for the indicated antibodies. **b** MDA-MB-231-ZEB1shRNA-G_{11} cells and MDA-MB-231-GAPDHshRNA cells were treated with DHT for 48 h

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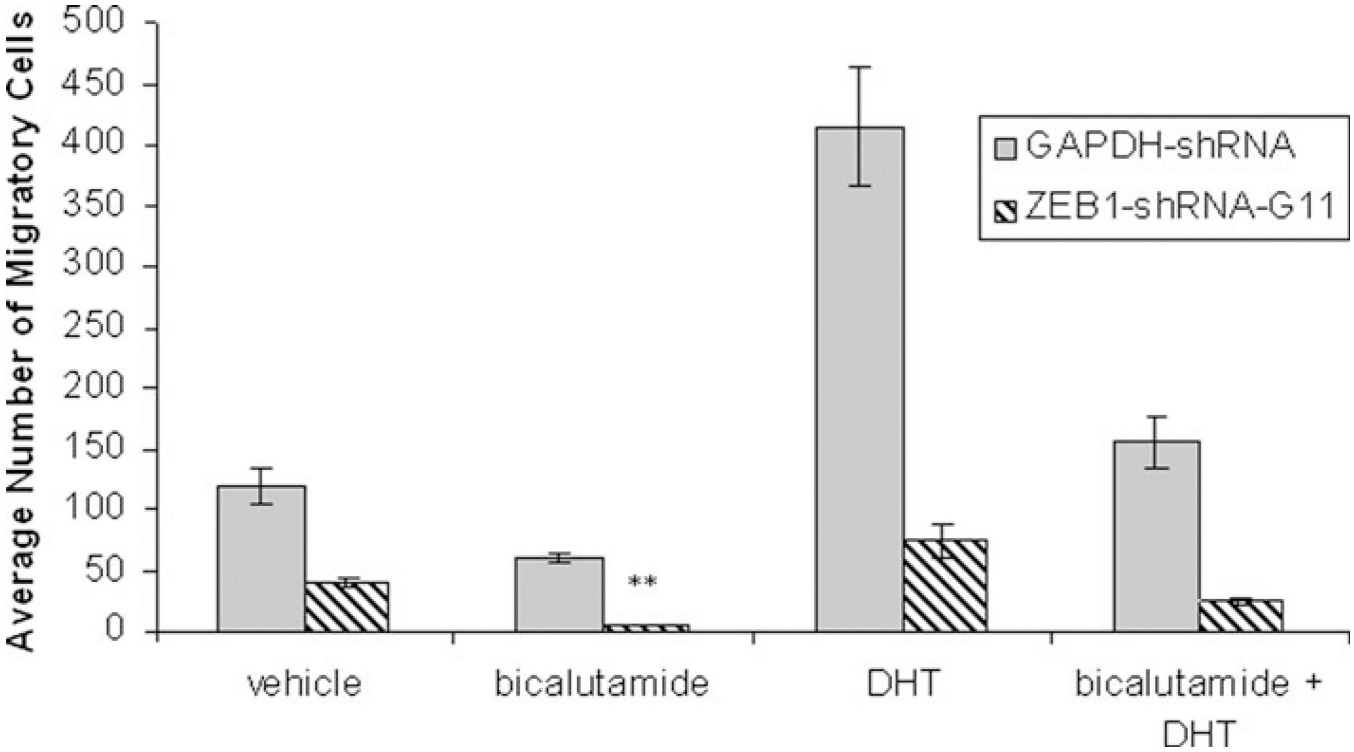

Fig. 5.

Migratory response of MDA-MB-231 cells to AR agonists and antagonists. Control transfected and ZEB1-shRNAG_{11} transfected cells were cultured in phenol red free medium for 24 h. 5×10^4 cells were added to the collagen coated inserts and treated with DHT (10 nM), bicalutamide (10 μM) or a combination for 6 h, $P<0.005$

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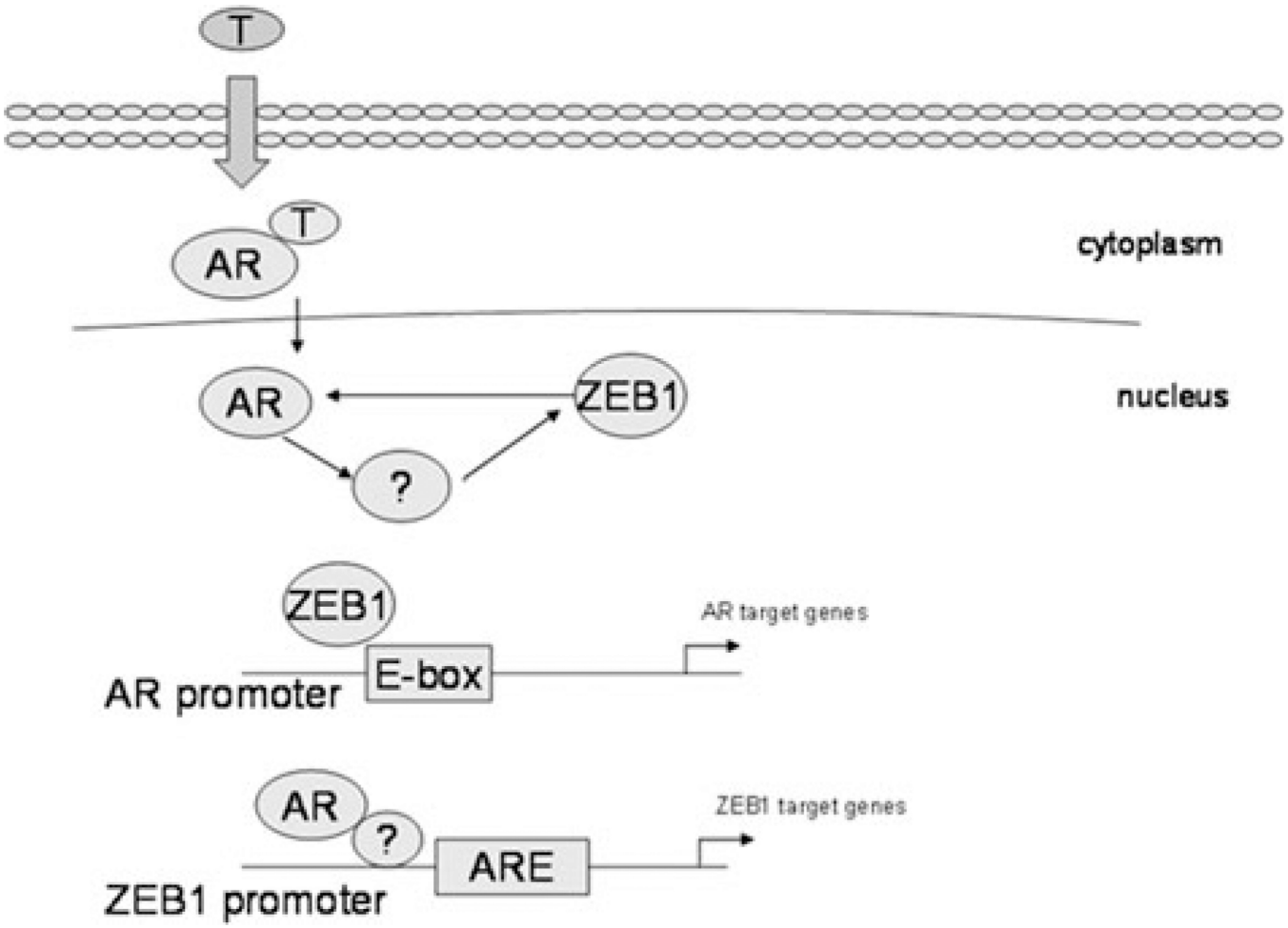

Fig. 6.

Proposed model for ZEB1-AR cross-talk. ZEB1 binds directly to the AR promoter to activate AR-regulated genes. AR regulates ZEB1 through an intermediary protein to modulate ZEB1 target genes

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Table 1

Immunohistochemical staining of ZEB1 and AR in breast cancer tissues

AR staining intensity scores	Number of ZEB1 positive tissues	
	ER+	ER−
0	20	5
1	38	13
2	9	32
3	3	10
ZEB1 staining intensity scores	Number of AR-positive tissues	
	ER+	ER−
0	21	9
1	30	11
2	13	23
3	6	17