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Suppression of estrogen receptor-alpha transactivation by thyroid transcription factor-2 in breast cancer cells

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ABSTRACT

Estrogen receptors (ERs), which mediate estrogen actions, regulate cell growth and differentiation of a variety of normal tissues and hormone-responsive tumors through interaction with cellular factors. In this study, we show that thyroid transcription factor-2 (TTF-2) is expressed in mammary gland and acts as ERα co-repressor. TTF-2 inhibited ERα transactivation in a dose-dependent manner in MCF-7 breast cancer cells. In addition, TTF-2 directly bound to and formed a complex with ERα, colocalizing with ERα in the nucleus. In MCF-7/TTF-2 stable cell lines, TTF-2 repressed the expression of endogenous ERα target genes such as pS2 and cyclin D1 by interrupting ERα binding to target promoters and also significantly decreased cell proliferation. Taken together, these data suggest that TTF-2 may modulate the function of ERα as a corepressor and play a role in ER-dependent proliferation of mammary cells.

1. Introduction

Estrogen receptors (ERs), ERα and ERβ, belong to the nuclear hormone receptor superfamily and mediate the actions of estrogen in the regulation of cell growth and differentiation in mammary glands [1,2]. In mice with a homozygous disruption of the ERα gene, the mammary glands remain undeveloped, indicating an indispensable role of ERα in the growth of mammary glands [3]. The fact that more than two-thirds of breast cancer patients are ERα-positive and benefit from antiestrogen or ovariectomy therapies also strengthens the importance of ERα in the stimulation of cell growth in mammary glands.

ERα consists of three functional domains [4]: an N-terminal region containing a constitutive activation function (AF-1); a central DNA-binding domain (DBD); and a C-terminal ligand-binding domain (LBD) containing a ligand-dependent activation function (AF-2). In the classic model of steroid hormone action, estrogen induces dimerization of ER, which is able to bind to estrogen response elements (EREs) in the promoters of ER target genes [5], such as pS2 [6], progesterone receptor (PR) [7], and cathepsin D [8]. Regulation of gene expression by ERα requires the interactions with cofactors including coactivators and corepressors. Most of the cofactors regulate ERα transcriptional activity in a ligand-dependent manner. However, some coactivators including cyclin D1 [9] and XBP-1 [10], and some corepressors including BRCA1 [11] regulate ERα transactivation in a ligand-independent manner. BRCA1 regulates both estrogen-dependent and -independent ERα transactivation [12].

Thyroid transcription factor-2 (TTF-2) is a member of the forkhead/winged helix family of transcription factors [13,14] and is a promoter-specific transcriptional repressor [14,15]. TTF-2 is expressed in endoderm lining the foregut, ectoderm that gives rise to the anterior pituitary, hair follicles and prepubertal testis, as well as the thyroid [16,17]. Expression and function of TTF-2 may also occur in other tissues as well. For example, in humans, mutations of the gene encoding for TTF-2 result in the Bamforth–Lazarus syndrome, characterized by thyroid agenesis, cleft palate, spiky hair and choanal atresia [18]. In the thyroid, TTF-2 regulates its differentiation through negative control of the expression of thyroglobulin (Tg) and thyroperoxidase (TPO) genes. Although mainly acting as a regulator of the expression of thyroid-specific genes, TTF-2 may act as a transcriptional regulator of other genes as well.

In the present study, we demonstrate that TTF-2 is expressed in mammary glands and its expression is decreased during late pregnancy in mice. TTF-2 physically interacts with ERα and interrupts ERα binding to target promoters, thus repressing ERα transactivation in breast cancer cells. Furthermore, TTF-2 inhibits cell growth in breast cancer cells. Taken together, these data suggest that TTF-2 may function as a corepressor of ERα and modulate the proliferation of mammary cells. As far as we know, this is the first study

Abbreviations: TTF-2, thyroid transcription factor-2; ERα, estrogen receptor-alpha; ERE, estrogen response element; DOX, doxycycline; E2, 17β-estradiol.

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to explore the role of TTF-2 in mammary cells, and to suggest it as a nuclear receptor corepressor.

2. Materials and methods

2.1. Animals

Sprague Dawley (SD) rats and ICR mice were purchased from Daehan Laboratories in Korea. Animals were maintained in a controlled environment with a 12 h light/dark cycle at 23 °C with food and water available *ad libitum*, and handled according to the NIH Guide for the Care and Use of Laboratory Animals.

2.2. Plasmids

Plasmids for mammalian expression and in vitro translation of ERα, ERβ, and TTF-2 were constructed using a mammalian expression vector, pCDNA3 (Invitrogen, CA). Full length and mutants of GFP–TTF-2 expression plasmids and N-terminal and FHD regions of GST–TTF-2 have been previously described [19]. Full length and C-terminal regions of GST–TTF-2 were constructed by subcloning the corresponding DNA fragments into the EcoRI–XhoI site of pGEX-4T (Amersham Pharmacia, Sweden).

2.3. Cell culture and transient transfection assay

MCF-7 and COS-7 cells were maintained in Dulbecco's minimum essential medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen). Cells were cultured at 37 °C in 5% CO_2 humidified atmosphere. For starvation, cells were plated in 24-well plates in DMEM containing 5% charcoal-stripped FBS and transfected with the indicated amount of expression plasmids, the reporter ERE-luc and the control β-gal expression plasmid pRSV using Superfect reagent (Qiagen, Germany). Total amounts of expression vector were kept constant by adding appropriate amounts of pcDNA3 empty vector. Cells were treated with either 100 nM 17β-estradiol (E2) or vehicle for 24 h. Luciferase and β-galactosidase activities were assayed as previously described [20]. The levels of luciferase activity were normalized to β-gal expression.

2.4. GST pull-down assay

GST pull-down assay was conducted as previously described [20]. Bacterially produced and immobilized GST fusion proteins, GST–TTF-2 and GST–TTF-2 mutants, were incubated with [^{35}S] methionine-labeled proteins, ERα or ERα deletion mutants. Bound proteins were washed and then analyzed by SDS–PAGE and autoradiography.

2.5. Establishment of stable cell lines

To establish stable cell lines for the inducible expression of TTF-2, we used the Tet-On-inducible system [21]. MCF-7 cells were cotransfected with pUHDrtTA2S-M2 (TRE) (a gift from Dr. H. Bujard, Heidelberg University, Germany) and pBI–EGFP–TTF-2 or pBI–EGFP (Clontech). Next day after transfection, cells were selected in culture media containing 800 μg/ml G418 (Invitrogen) for 2 weeks.

2.6. Co-immunoprecipitation and Western blot analysis

Co-immunoprecipitation assays were performed with COS-7 cells transfected with pCDNA-HA–ERα and GFP–TTF-2 plasmids in DMEM containing 5% charcoal-stripped FBS. Transfected cells were treated with 100 nM E2 for 3 h and harvested with RIPA cell lysis buffer. Whole-cell lysate was incubated with anti-GFP antibody (sc-8334, Santa Cruz Biotechnology) and subsequently with protein A-agarose bead slurry (Invitrogen). After washing beads with RIPA buffer at 4 °C, bound proteins were separated by SDS–PAGE and subjected to Western blot analysis. Signals were then detected using an ECL kit (Amersham Pharmacia).

2.7. Immunofluorescence

MCF-7 cells were transfected with GFP–TTF-2 (1–376) or GFP–TTF-2 (152–376) together with ERα plasmids and treated with 100 nM E2 for 24 h. Cells were washed with PBS and fixed with 2% paraformaldehyde for 15 min. For detection of ERα, cells were subsequently incubated with primary anti-ERα antibody, biotin-conjugated goat anti-rabbit secondary antibody (Zymed) and TRITC–streptavidin-conjugated enzyme (Zymed). Stained cells were mounted on glass slides and observed under a laser scanning confocal microscope (Leica TCS, Heerbrugg, Switzerland).

2.8. Northern blot analysis and RT-PCR

Northern blot analysis was performed as previously described [20]. For RT-PCR, total RNA was reverse-transcribed and PCR-amplified with TTF-2-specific primers (forward: 5′-CCGTGAAG-GAAGAGCGCGG-3′ and reverse: 5′-GGTCCGAGCGCTTGAAGCG-3′) in ORF. As a control, PCR reactions were also performed using β-actin-specific primers (forward: 5′-GAGACCTTCAACACCCCAGCC-3′ and reverse: 5′-CCGTCAGGCAGCTCATAGCTC-3′) in exon 4.

2.9. Electrophoretic mobility shift assay

GST fusion proteins were expressed in *Escherichia coli* BL21 cells and purified with glutathione-Sepharose-4B beads. Gel mobility shift assay was performed as previously described [20]. The DNA sequence of ERE oligonucleotide is as follows (ERE sequence is underlined): 5′-GGCCAAAGTCAGGTCACAGTGACCTGATCA-3′ (forward) and 5′-GGCCTGATCAGGTCACTGTGACCTGACTTT-3′ (reverse).

2.10. Chromatin immunoprecipitation assay

MCF-7/TTF-2 cells starved for 48 h were treated with 500 ng/ml DOX for 30 h and then treated with 10 nM E2 for 1 h. Cells were cross-linked with 1% formaldehyde and performed ChIP assay as previously described [20]. Briefly, anti-ERα antibody (ab75635, Abcam) was used for immunoprecipitation. Immunoprecipitated DNA and input-sheared DNA were subjected to PCR using a pS2 promoter primers (forward: 5′-CCAGGCCTACAATTTCATTAT-3′ and reverse: 5′-AGGGATCTGAGATTCAGAAAG-3′), which amplify the region containing ERE. As a control, PCRs were performed using GAPDH specific primers (forward, 5′-TTCATTGACCTCAACTACATG-3′ and reverse, 5′-GTGGCAGTGATGGCATGGAC-3′).

2.11. MTS assay

Mock and MCF-7/TTF-2 cells (2000 cells/well) were plated in phenol red-free DMEM containing 10% FBS in 96 well plates and were treated with 1 μg/ml DOX. Medium was changed every 2 days. At the end of the incubation, cell proliferation was determined by the Celltiter 96 Aqueous nonradioactive proliferation (MTS) assay (Promega).

3. Results

3.1. TTF-2 is expressed in mammary glands and represses ERα transactivation

TTF-2 is expressed in several mouse tissues, including the pituitary gland, hair follicles, and prepubertal testis, with the highest level of expression in the thyroid [16,17]. To explore whether TTF-2 is also expressed in other organs, which has been previously untested, the expression of TTF-2 mRNA was detected in several rat tissues by RT-PCR. As shown in Fig. 1A, TTF-2 was expressed in the epididymis and mammary glands. Interestingly, TTF-2 expression in mammary glands was observed in a stage-dependent manner during mouse pregnancy (Fig. 1B). The expression level of TTF-2 was high during virginity and the early stage of pregnancy, such as E7.5, but was down-regulated during the mid-late stages of pregnancy, at which mammary epithelium is highly proliferated by ERα signaling. In breast cancer cell line, TTF-2 was expressed in ERα-negative MDA-MB-435 cells, but was not expressed in ERα-positive MCF-7, T47D, and ZR-75-1 cells (Fig. 1C). Together, these results suggest that TTF-2 is expressed in mammary glands, and that its expression may be related to ERα signaling.

To examine whether TTF-2 affects ERα transactivation, transient transfection assays were performed in MCF-7 cells, ER-positive human breast cancer cells. TTF-2 inhibited ER transactivation in a dose-dependent manner (Fig. 1D). Because both ERα and ERβ are endogenously expressed in MCF-7 cells, we examined the specificity of TTF-2 for ERα by transient transfection assays in ER-deficient COS-7 cells. The transactivation of exogenous ERα, but not ERβ, was down-regulated by TTF-2 (Fig. 1E). Interestingly, TTF-2 also inhibited ERα transactivation in the absence as well as in the presence of E2 (Fig. 1F). However, TTF-2 has no effect on the transactivation of other classical receptors, retinoid X receptor (RXR) and androgen receptor (AR) (Fig. 1G). These results suggest that TTF-2 specifically inhibits ERα-mediated transactivation in both estrogen-dependent and independent manners.

3.2. The C-terminal region of TTF-2 is responsible for the repression of ERα transactivation through physical interaction

To investigate whether the functional interaction between TTF-2 and ERα occurs through their physical interaction, we performed co-immunoprecipitation assays. The results showed that TTF-2 was co-precipitated with ERα (Fig. 2A). The region of each protein responsible for the interaction between TTF-2 and ERα were then assessed by glutathione-S-transferase (GST) pull-down assays. TTF-2 interacted with ERα (ΔAF-2), ERα (AF1 + DBD + h), ERα (LBD + AF-2), and ERα-AF-1, as well as full-length ERα in a ligand-independent manner (Fig. 2B). These results suggest that two regions of ERα, 123–206 and 340–423 amino acid residues, which are a part of the AF-1 and LBD region, respectively, are important for its binding with TTF-2. Meanwhile, ERα interacted with all mutants of TTF-2 in a ligand-independent manner (Fig. 2B). Taken together, these results suggest that TTF-2 physically interacts with ERα through the AF-1 and LBD regions of ERα and multiple domains of TTF-2.

The region of TTF-2, which affects ERα transactivation, was accessed by transient transfection assays with expression plasmids of GFP–TTF-2 mutants and ERE-luc reporter. As shown in Fig. 2C, the C-terminal and FHD (forkhead domain) regions of TTF-2, but not N-terminal region, significantly inhibited ERα transactivation in MCF-7 cells, although the repressive activity of the C-terminal region of TTF-2 was stronger than the FHD region. The previous study reported that the full-length and the FHD region of TTF-2, containing a nuclear localization signal (NLS), exclusively localizes in the nucleus; however, the C-terminal region of TTF-2, containing no NLS, localizes diffusely in both the cytoplasm and nucleus [19]. As expected, the C-terminal region of TTF-2 as well as the full-length colocalized with ERα in the nucleus (Fig. 2D). These results suggest that the C-terminal region of TTF-2 may be mainly responsible for the repression of ERα transactivation, although ERα interacts with multiple regions of TTF-2.

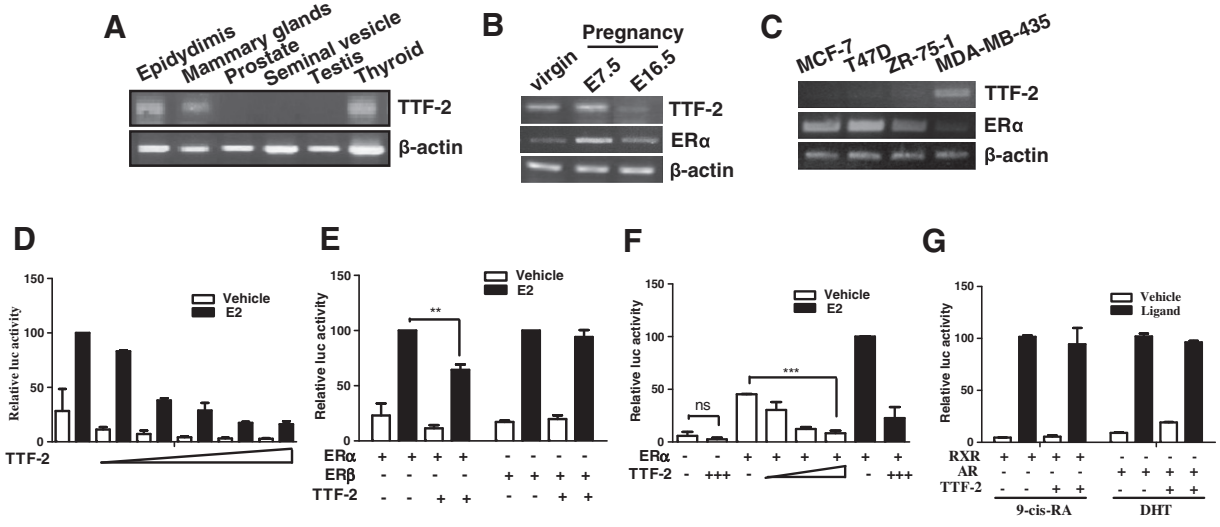

Fig. 1. Expression of TTF-2 in mammary glands and TTF-2 repression of ERα transactivation. (A–C) The expression level of TTF-2 mRNA was detected in various rat tissues, mammary glands at two developmental stages during mouse pregnancy, and breast cancer cell lines by RT-PCR. (D) Dose-dependent inhibition of ERα transactivation by TTF-2. MCF-7 cells were transiently transfected with ERE-luc reporter, along with increasing amounts (5, 10, 20, 60, and 100 ng) of TTF-2 expression plasmid. Luciferase activity was measured 24 h after the treatment with vehicle (EtOH) or 100 nM 17β-estradiol (E2). (E) Specific inhibition of ERα transactivation by TTF-2. COS-7 cells were transiently transfected with TTF-2 expression plasmid, ERE-luc reporter, and ERα or ERβ expression plasmids. (F) E2-independent inhibition of ERα transactivation by TTF-2. COS-7 cells were transiently transfected with ERα and increasing amounts of TTF-2 expression plasmids, along with ERE-luc reporter. (G) Effect of TTF-2 on the transactivation of other nuclear receptors. COS-7 cells were transiently transfected with TRE-luc and RXR or ARE2–TATA-luc and AR. Luciferase activity was measured 24 h after the treatment with 100 nM 9-cis-ratinoic acid (9-cis-RA) or 10 nM DHT. The error bars indicate the standard errors of the means (SEM). Each experiment was repeated at least three times in duplicate. The error bars indicate the standard error of the mean. $^{**}P < 0.01$; $^{***}P < 0.001$; ns, not significant.

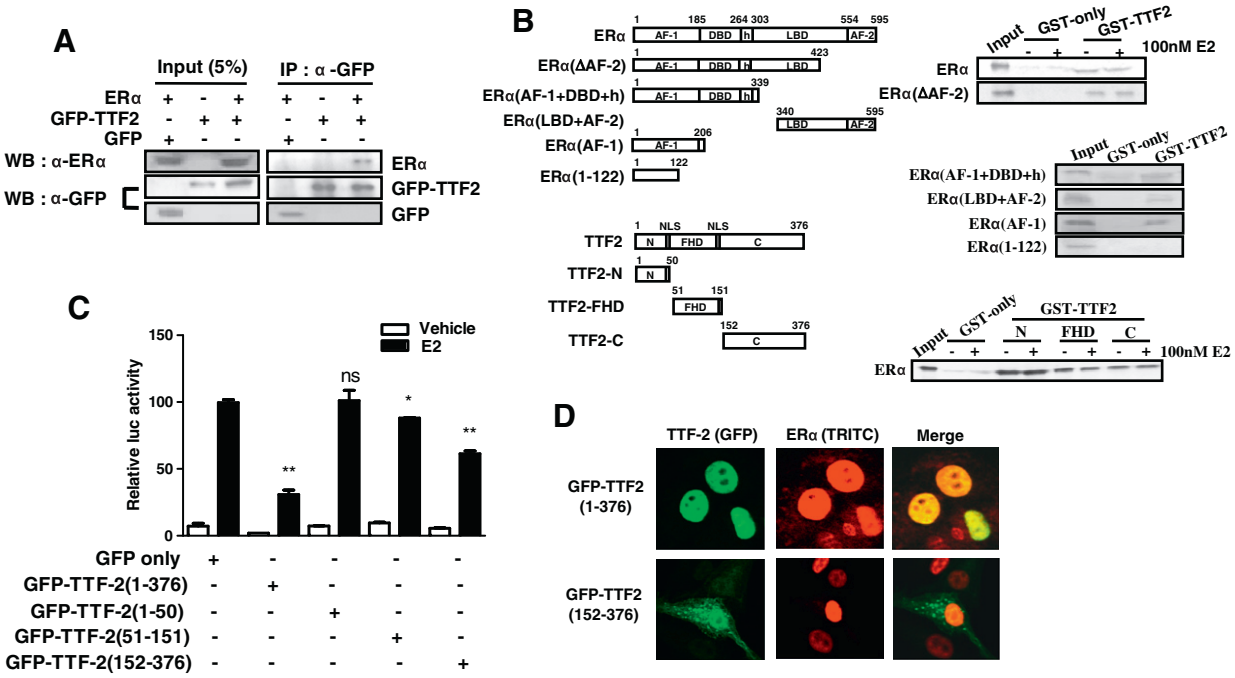

Fig. 2. Physical association of TTF-2 with ERα. (A) Physical interaction between TTF-2 and ERα. COS-7 cells were transiently transfected with GFP–TTF-2 and ERα expression plasmids, and treated with 100 nM E2 for 24 h post-transfection. Co-immunoprecipitations were conducted with anti-GFP antibody. (B) Domains responsible for the direct interaction between TTF-2 and ERα. Schematic representation of the full-length and deletion mutants of ERα and TTF-2 are shown. GST alone or GST fusion proteins of TTF-2 immobilized to glutathione-agarose beads were incubated with equivalent amounts of the ^{35}S-labeled ERα in the presence or absence of E2. (C) The effect of TTF-2 domains on ERα transactivation. MCF-7 cells were transiently transfected with ERE-luc reporter and an expression plasmid of GFP–TTF-2 deletion mutants as indicated. The error bars indicate the standard error of the mean. $^{*}P < 0.05$; $^{**}P < 0.01$; ns, not significant in comparison of indicated bars versus the second bar, GFP only with E2 treatment. (D) Colocalization of ERα and TTF-2 in MCF-7 cells. MCF-7 cells were transiently transfected with GFP–TTF-2 (1–376) or GFP–TTF-2 (152–376) expression plasmids, treated with 100 nM E2 for 24 h, and processed for immunocytochemistry using anti-ERα antibody.

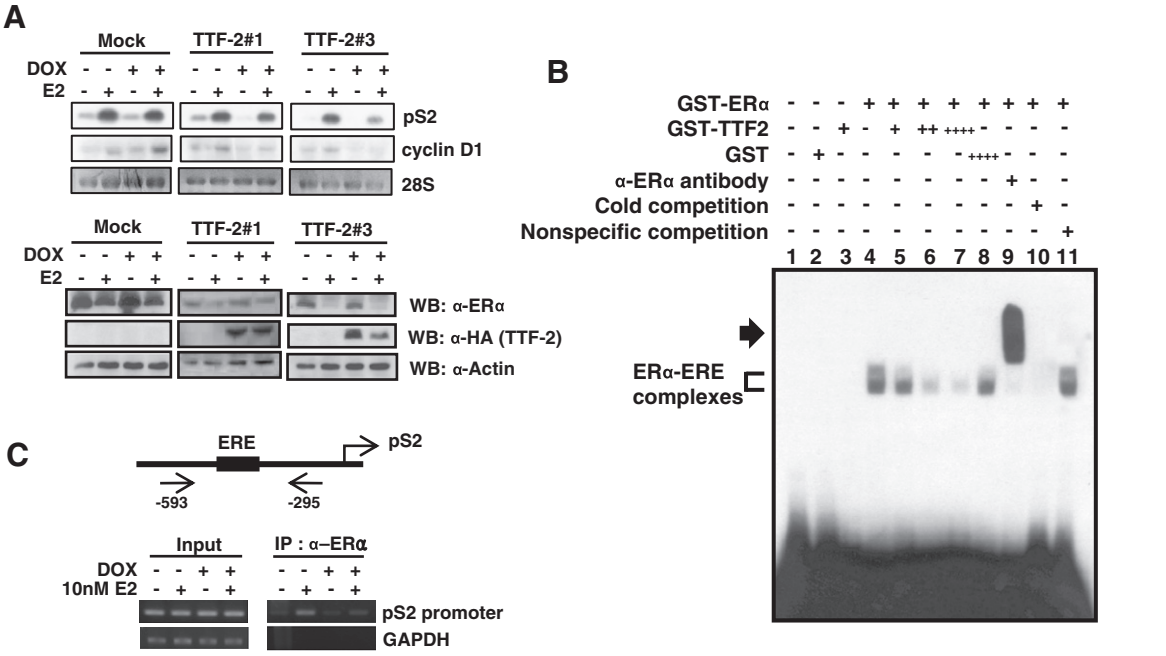

Fig. 3. Inhibition of the expression of ERα target genes by TTF-2. (A) Upper panel: Down-regulation of E2-induced pS2 and cyclin D1 mRNA levels by TTF-2 overexpression in MCF-7/TTF-2 cells. Cells were treated with or without 500 ng/ml DOX for 12 h and then treated with vehicle or 100 nM E2 for 24 h. mRNA levels were analyzed by Northern blot analysis. Lower panel: No change of ERα protein levels in MCF-7/TTF-2 cells. The protein level was determined using anti-HA antibody (HA-TTF-2) and anti-ERα antibody. (B) Interference with the formation of ERα-DNA complex by TTF-2. ^{32}P-labled ERE probe was incubated with GST–ERα and GST–TTF-2 proteins (ratios from 1:1 to 1:4) in EMSA binding buffer and analyzed on a polyacrylamide gel. A 50-fold molar excess of unlabeled ERE probe was added as a cold competitor (lane 10). Unlabeled ARE probe was added as a nonspecific competitor (lane 11). The migration positions of the supershifted band formed by Ab-ERα–ERE are indicated with an arrow. (C) Inhibition of ERα recruitment to the pS2 gene promoter in MCF/TTF-2 cells. Cells were treated with 500 ng/ml DOX for 30 h and then treated with 10 nM E2 for 1 h. Cells were processed for ChIP assay using anti-ERα antibody.

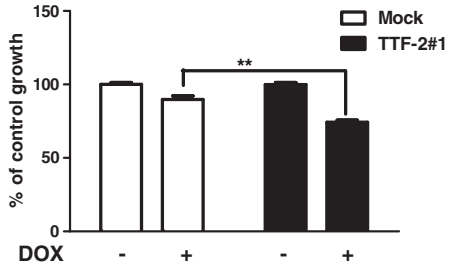

Fig. 4. Inhibition of cell proliferation by TTF-2 in breast cancer cells. Mock and MCF-7/TTF-2 cells were treated with or without 1 μg/ml DOX for 5 days to induce the expression of TTF-2. Cell proliferation was measured by MTS assays. Values represent the standard errors of the means (SEM) of six independent wells of cells from two independent cultures. $^{**}P < 0.01$.

3.3. TTF-2 inhibits the expression of ERα target genes by interfering with ERα binding to target promoters

In order to elucidate the biological function of TTF-2 on ERα-mediated signaling, we established stable MCF-7/TTF-2 cell lines, in which the expression of HA-tagged TTF-2 was driven by a Tet-On promoter [21]. Overexpression of TTF-2 by DOX treatment down-regulated E2-induced pS2 and cyclin D1 mRNA levels in MCF-7/TTF-2 cell lines (Fig. 3A, upper panel). Levels of ERα protein were not significantly affected by DOX treatment, although ERα protein levels decreased with 17β-estradiol (E2) treatment as previously reported [22] (Fig. 3A, lower panel). Interestingly, E2 treatment decreased the levels of TTF-2 protein, as well as ERα in MCF-7/TTF-2 cell lines. These results strongly support that TTF-2 regulates ERα transactivation and thus the expression of endogenous ERα target genes.

To explore the molecular mechanisms by which TTF-2 suppresses ERα transactivation, we tested the effect of TTF-2 on ERα protein level, translocation, and DNA binding activity, as well as the possibility of histone deacetylase (HDAC) recruitment and competition with ERα coactivators by TTF-2. We found that TTF-2 neither recruited HDAC nor competed with ERα coactivators (data not shown). It also did not influence ERα protein level (Fig. 3A) and nuclear translocation (Fig. 2D). However, analysis by EMSA showed that TTF-2 altered the DNA binding activity of ERα (Fig. 3B). Binding of ^{32}P-labeled ERE to GST–ERα protein resulted in the formation of ERα–ERE complexes, a monomer and homodimer complex of ERα (Fig. 3B, lane 4). The formation of ERα–ERE complexes decreased in response to increasing concentration of GST–TTF-2 protein (Fig. 3B, lanes 5–7).

To further confirm that TTF-2 affects the recruitment of ERα to target promoters, ChIP assay was performed using MCF-7/TTF-2 cells. As expected, the expression of TTF-2 inhibited E2-induced recruitment of ERα to the pS2 gene promoter (Fig. 3C). These results suggest that the repression of ERα transactivation by TTF-2 is at least a result of its interference with ERα binding to DNA.

3.4. TTF-2 suppresses the proliferation of breast cancer cells

To investigate the effect of TTF-2 on cell proliferation, we performed MTS assays using MCF-7/TTF-2 cells. As shown in Fig. 4, overexpression of TTF-2 by DOX treatment significantly inhibited the growth of MCF-7 cells. These results suggest that TTF-2 expression inhibits the proliferation of ERα-positive breast cancer cells.

4. Discussion

Findings showing that TTF-2 expression is regulated in mammary gland and breast cancer cell lines and suppresses ERα transactivation (Fig. 1) suggest a novel biological function of TTF-2 in ERα-mediated cell proliferation. Previous studies have suggested a role of TTF-2 as a promoter-specific transcriptional repressor [14,15]. In this study, we found that TTF-2 was capable of interacting with ERα and regulating ERα-mediated transcription in breast cancer cells, implying TTF-2 as a corepressor of ERα. Interestingly, TTF-2 repressed ERα transactivation in a ligand-independent manner as well as a ligand-dependent manner. TTF-2 might repress the transactivation of unliganded ERα through direct binding with unliganded ERα bound to promoters. Such a phenomenum was previously reported with BRCA1, which interacted with and repressed the transactivation of DNA-bound unliganded ERα [12].

The expression level of ERα corepressors has been shown to be related to normal development and tumorigenesis of mammary glands [23,24]. Metastasis-associated protein (MTA-1), a known ERα corepressor, which is expressed in normal mammary glands, is down-regulated during pregnancy and is up-regulated in breast tumors [25]. Moreover, the deregulation of MTA-1 causes inappropriate development of mammary glands, such as increased cell proliferation, hyper-branched ductal structure, precocious development, and tumorigenesis [24]. On the other hand, the expression of N-CoR, another ERα corepressor, is up-regulated during the development of carcinomas from normal mammary glands and is decreased during the progression of breast cancer [23]. In this study, we showed that the expression of TTF-2 decreased during the stage of ERα-dependent proliferation of mammary glands, E16.5, in mice (Fig. 1). In addition, TTF-2 overexpression downregulated cyclin D1 expression, a regulator of the cell cycle (Fig. 3B) and inhibited cell proliferation in breast cancer cells (Fig. 4). Therefore, TTF-2 may be an important regulator of ERα-mediated proliferative action in mammary gland cells [26].

During thyroid development, TTF-2 represses the transcriptional activity of TTF-1 and Pax-8 through its C-terminal region, which contains an alanine-rich domain [14,15]. This study showed that TTF-2 also repressed ERα transactivation through its C-terminal region (Fig. 2C). Previous studies have reported that the C-terminal region, which contains an alanine-rich domain in some DNA-binding proteins, including Fifth Ewing Variant (FEV) [27], TBP-binding protein Dr1 [28], and homeobox protein EVX1 [29] is responsible for the activity of transcriptional repression. Therefore, the C-terminal region of TTF-2 may generally act as a transcriptional repressor domain in modulation of the transcriptional activity of some transcription factors, although it does not directly bind to DNA.

In conclusion, we have found that TTF-2 physically interacts with ERα *in vivo* and functions as a corepressor in regulating ERα-mediated signaling in mammary cells. These findings may provide insight into the understanding of ERα-mediated development of mammary glands and also ERα-mediated development and progression of breast cancers.

Acknowledgments

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