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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).