Nuclear tristetraprolin acts as a corepressor of multiple steroid nuclear receptors in breast cancer cells

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Abbreviations: TTP, tristetraprolin; ERα, estrogen receptor; PR, progesterone receptor; AR, androgen receptor; GR, glucocorticoid receptor.

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Abstract

Tristetraprolin (TTP) is a 34-kDa, zinc finger-containing factor that in mammalian cells acts as a tumor suppressor protein through two different mechanisms. In the cytoplasm TTP promotes the decay of hundreds of mRNAs encoding cell factors involved in inflammation, tissue invasion, and metastasis. In the cell nucleus TTP has been identified as a transcriptional corepressor of the estrogen receptor alpha (ERα), which has been associated to the development and progression of the majority of breast cancer tumors. In this work we report that nuclear TTP modulates the transactivation activity of progesterone receptor (PR), glucocorticoid receptor (GR) and androgen receptor (AR). In recent years these steroid nuclear receptors have been shown to be of clinical and therapeutical relevance in breast cancer. The functional association between TTP and steroid nuclear receptors is supported by the finding that TTP physically interacts with ERα, PR, GR and AR in vivo. We also show that TTP overexpression attenuates the transactivation of all the steroid nuclear receptors tested. In contrast, siRNA-mediated reduction of endogenous TTP expression in MCF-7 cells produced an increase in the transcriptional activities of ERα, PR, GR and AR. Taken together, these results suggest that the function of nuclear TTP in breast cancer cells is to act as a corepressor of ERα, PR, GR and AR. We propose that the reduction of TTP expression observed in different types of breast cancer tumors may contribute to the development of this disease by producing a dysregulation of the transactivation activity of multiple steroid nuclear receptors.

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the proliferation rate and tumorigenicity potential of human breast cancer cells in mice [4]. These results allowed us to suggest that nuclear TTP also acts as a tumor suppressor protein through a mechanism that involves its ability to regulate ERα transactivation [4].

In this work, we sought to gain better insight into the function of nuclear TTP as a transcriptional corepressor by exploring its effect on the activity of other hormone nuclear receptors that have been recently shown to be of clinical or therapeutic relevance in breast cancer [22–25]. Our results show that TTP is physically associated to progesterone receptor (PR), glucocorticoid receptor (GC) and androgen receptor (AR) in the nucleus of breast cancer MCF-7 cells in vivo. We show further that TTP overexpression reduces ERα, PR, GC and AR transactivation activity. In contrast, siRNA-mediated reduction of endogenous TTP protein levels produced a significant increase in the transcriptional activity of these steroid nuclear receptors without affecting their protein levels in MCF-7 cells. These results suggest that the corepressor activity of nuclear TTP may participate in the regulation of multiple hormone-dependent signaling pathways that play a key role in the development of human normal mammary gland epithelium and breast cancer tumors.

2. Materials and methods

2.1. Reagents and Antibodies

Estradiol (E8875, 17β-estradiol), progesterone (P-0130, 4-Pregnen-3-20 dione) and dexmethasone (D1756), were obtained from Sigma-Aldrich. Androgen (10300, 5α-Pregnene-3,20 dione) and dexamethasone (D1756), were obtained from Sigma-Aldrich. Androgen receptor (AR) in the nucleus of breast cancer MCF-7 cells was obtained from Fluka Chemika. Human ERα antibody (Sc D-12, Sc HIS-20) and Human TTP antibody (Sc-12565) were purchased from Santa Cruz Biotechnology, Inc. TTP polymer (T5327) antibody was obtained from Sigma-Aldrich. Human PR (ab-68195), GR (ab-2768) and AR (ab-74272) antibodies were from Abcam. TTP knockdown assays were performed using TTP siRNA mixture and control siRNA from Santa Cruz Biotechnology and transfected using Lipofectamine 2000 (Invitrogen). The specificity of the TTP knockdown assay was determined by analyzing the expression of TTP, ERα, PR, GC and AR in MCF-7 cells transfected with a control siRNA or with a specific siRNA-TTP.

2.2. Plasmids

ERα transactivation activity was determined using a luciferase reporter gene driven by a promoter containing three estrogen responsive elements (ERE-Tk-LUC) that has been previously described [4,26]. To test the transactivation activity of PR, AR, GC and GR, human cells were transfected with the PGL3-MMTV in which the luciferase reporter gene is regulated by a promoter containing a palindromic sequence recognized by PR, GC and GR. pcdNA3.1-ERα and ERE-Tk-LUC vectors were kindly provided by Dr. W. Lee Kraus (Cornell University). Vectors PRE-dbCAT (PR), pSVhARo-BHEXE (AR), pcdNA3.1-GR and PGL3-MMTV were a gift from Dr. Rocío Ángeles García Becerra (Instituto Nacional de Ciencias Médicas y Nutrición–México). Human full-length TTP cDNA (GenBank™ accession no. NM_0034073.1) was cloned into the mammalian expression vector pCMV-3Tag-1 A (Agilent Technologies, Santa Clara, CA) as previously described [4]. The sequences of all constructs were verified by DNA sequencing at LARAGEN Inc. (Culver City, CA).

2.3. Cell culture and transfection assays

MCF-7 cells (ATCC HTB-22, American Type Culture Collection, Manassas, VA) were maintained in Minimum Essential medium Alpha Medium (11900-024, Gibco) supplemented with 5% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin in a humidified atmosphere containing 5% CO2 at 37 °C. Cells were seeded into tissue culture dishes containing phenol red-free Minimum Essential Medium Eagle (M3024, Sigma-Aldrich) supplemented with 5% charcoal/dextran-treated FBS and cultured for 36–40 h before all experimental treatments with hormone. Cells were transfected using Lipofectamine 2000 with 2 mg of ERE-Tk-LUC reporter, 1 mg of pcDNA3.1-ERα, PR, GC, AR and 0.5 mg pCMV-3Tag-1 A-TTP or 0.006 mg of Renilla vector (transfection control). After 4 h, the cells were washed twice with phosphate-buffered saline and treated with 100 nM of estradiol, progesterone, dexmethasone or androgens for 48 h in phenol red-free MEM supplemented with 5% stripped FBS. Cells were then washed and harvested in Passive Lysis Buffer (Promega). Control cells were incubated under the same experimental conditions in the absence of hormones. Luciferase and Renilla activities were measured using a Dual-Luciferase Reporter Assay System (E1960, Promega) and Glomax Multi JR Detection System (Promega). TTP knockdown assays were performed using TTP siRNA mixture and control siRNA from Santa Cruz Biotechnology and transfected using Lipofectamine 2000 (Invitrogen). The specificity of the TTP knockdown assay was determined by analyzing the expression of TTP, ERα, PR, GC and AR in MCF-7 cells transfected with a control siRNA or with a specific siRNA-TTP.

2.4. Immunoprecipitation and Western blot

MCF-7 cells were lysed with TNE buffer (50 mM Tris–HCL, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.5% Triton X-100 plus a mixture of protease and phosphatase inhibitors). Proteins were immunoprecipitated with mouse anti-ERα, anti-GR or rabbit anti-PR and anti-AR antibodies. Immunoprecipitated proteins were separated by SDS-PAGE, and Western blot analysis was performed using a specific antibodies anti-TTP and anti-mouse or anti-rabbit secondary HRP-conjugated antibody (Pierce). Proteins were visualized using an enhanced chemiluminescence assay (SuperSignal West Pico Chemiluminescent Substrate, Thermo Scientific).

2.5. Statistical analysis

Each transfection assay was performed by triplicate in three different experiments using different cell cultures. Data are presented as mean ± S.E. Statistical significance was analyzed at 0.05 levels of significance using Student’s t-test.

3. Results

3.1. Correlation between TTP mRNA expression levels and breast carcinoma using the Oncomine cancer microarray database

In this study, we set out to determine the effect of TTP on the transactivation activity of the steroid nuclear receptors PR, GC and AR that along with ERα have been associated to cell proliferation, tumor development, apoptosis control and response to hormonal therapy in breast cancer. To explore the relationship between TTP expression and breast cancer tumorigenesis, we compared TTP mRNA levels in normal tissues and different types of breast carcinomas using the Oncomine database (http://www.oncomine.org, Compendia Bioscience, Ann Arbor, MI). The analysis of data sets containing gene chip profiles classified as normal or breast carcinoma tissues, showed a significant reduction in TTP mRNA levels in breast carcinomas compared to normal tissue. Two representative results of two independent data sets [27] are shown in Fig. 1.

3.2. TTP interacts with different steroid nuclear receptors in MCF-7 cells in vivo

To study the functional interaction between TTP and steroid nuclear receptors we used breast cancer MCF-7 cells that had previously shown to express multiple steroid nuclear receptors [25,28–36]. To explore the physical interaction between TTP and the steroid nuclear receptors ERα, PR, GC and AR, we performed coimmunoprecipitation assays in MCF-7 cells. Endogenous nuclear hormone receptors present in MCF-7 protein
3.3. TTP represses ERα, PR, GR and AR transcriptional activity

To test whether TTP expression affects PR, GR and AR transactivation we performed transient transfection assays in MCF-7 cells. In these experiments, pCDNA3.1–TTP was the source of TTP, and the luciferase reporter vectors ERE-Tk-LUC and MMTV-LUC were used as indicators of ERα, PR, GR and AR transcriptional activity as described in materials and methods. Endogenously expressed ERα, PR, GR and AR showed baseline luciferase activity in MCF-7 cells that increased upon stimulation with their respective hormone ligands (Fig. 3A–D). As previously reported [4] transfection of MCF-7 cells with 250 ng of pCMV-3Tag-1 A–TTP in the presence of 17β-estradiol reduced the transcriptional activity of ERα by 73% (Fig. 3A). The same treatment reduced the ligand-dependent transactivation of PR, GR and AR by 93%, 77% and 76%, respectively (Fig. 3B–D). These results suggest that TTP acts as a common transcriptional corepressor for ERα, PR, GR and AR in MCF-7 cells.

Next, we used siRNA-mediated knockdown assays to mimic the TTP loss of expression observed in different carcinomas and to determine the functional relevance of this factor in the transactivation activity of endogenously expressed ERα, PR, GR and AR in MCF-7 cells. The siRNA-mediated reduction of TTP expression was confirmed by WB analysis of protein extracts prepared from MCF-7 cells transfected with a specific TTP-siRNA or with an unrelated control siRNA. The results showed a significant reduction in TTP expression in cells transfected with TTP-siRNA compared to MCF-7 cells transfected with the control siRNA (Fig. 4A, TTP panel). Transfection of MCF-7 cells with TTP-siRNA did not affect the expression of ERα, PR, GR and AR, or the protein levels of tubulin that was used as an unrelated loading control protein (Fig. 4A). These results confirm the specificity of the siRNA on TTP expression and suggest that TTP does not affect the expression of ERα, PR, GR and AR. Next, we determined the hormone-dependent activity of ERα, PR, GR and AR in MCF-7 cells transfected with TTP-siRNA or the control siRNA. Our results showed that siRNA-mediated knockdown of TTP expression increased the hormone-dependent transactivation activity of all steroid nuclear receptors tested, compared to MCF-7 cells transfected with siRNA control (Fig. 4B). These results indicate that TTP is an important participant in the regulation of ERα, PR, GR and AR transactivation and suggest the possibility that loss of TTP expression observed in breast cancer tumors may cause a dysregulation of multiple steroid nuclear receptor-dependent signaling pathways.

4. Discussion

Functional studies performed in mammalian cells had previously shown that TTP function as a tumor suppressor is mediated by two different mechanisms that depend on its cellular localization [4,37]. In the cytoplasm, TTP promotes the decay of mRNAs associated to inflammation, angiogenesis, tissue invasion, and metastasis (53–58). The nuclear form of TTP acts as a transcriptional corepressor via its interaction with HDACs. However, while cytoplasmic TTP regulates the expression levels of hundreds of mRNAs, its nuclear regulatory function had only been found to modulate the transcriptional activity of p65/NF-kB and ERα [4,37].

In this work, we have substantially extended the known targets of nuclear TTP by demonstrating it corepresses the transactivation of the steroid nuclear receptors PR, GR and AR in breast cancer MCF-7 cells. The functional association of TTP with these steroid nuclear hormone receptors was confirmed by different experimental strategies. First, immunoprecipitation assays showed that endogenous TTP physically interacts with PR, GR and AR in MCF 7 cells in vivo. Second, transient transfection of TTP produced a significant reduction in the transactivation activity of PR, GR and AR. Third, siRNA-mediated reduction of endogenous TTP protein levels increased the transcriptional activity of all the steroid nuclear receptors tested. Further, reduction of...
endogenous TTP protein levels in MCF-7 cells did not affect the apparent abundance of ER, PR, GR and AR, suggesting that the mechanism responsible for TTP-mediated repression of steroid nuclear receptors transactivation does not involve its mRNA destabilizing activity.

The tumor suppressor activity of nuclear TTP in breast cancer cells has been attributed to its ability to repress the transcriptional activity of ERα, a ligand activated transcription factor that is associated to the growth and progression of 70% to 80% of all breast cancer tumors. However, the results obtained in this study suggest that TTP regulates the activity and functions of other steroid nuclear receptors that also play an important role in tumor development and may influence tumor response to treatment [38]. A recent report has shown that PR acts as an ERα associated protein that redirects the binding of ERα to different chromatin sites. The PR-ERα complex is responsible for a novel gene expression pattern that inhibits the estradiol-dependent proliferation of breast cancer cells. Interestingly, the PR-dependent reprogramming of ERα regulates the transcription of the ZFP36 gene which encodes TTP [39]. Other studies have shown that the nuclear receptor GR is present in 50% of invasive breast cancers and most of the breast cancer cell lines used in biomedical research.

![Fig. 2. TTP interacts with ERα, PR, GR and AR in vivo.](image)

Fig. 2. TTP interacts with ERα, PR, GR and AR in vivo. Endogenous ERα (A), PR (B), AR (C) and GR (C) were immunoprecipitated from protein extracts prepared from MCF-7 cells using specific antibodies. Immunoprecipitated proteins were resolved by PAGE, and the binding of TTP was visualized by Western blot. The input lane represents 10% of the protein extract used in the coimmunoprecipitation assays. IP: immunoprecipitation, WB: Western blot.

![Fig. 3. TTP overexpression represses ERα, PR, GR and AR transactivation in MCF-7 cells.](image)

Fig. 3. TTP overexpression represses ERα, PR, GR and AR transactivation in MCF-7 cells. Human breast cancer MCF-7 cells were transiently transfected with empty pCMV-3TAG vector (control) or with 0.25 μg of pCMV-3TAG-TTP (FLAG-TTP) along with the corresponding luciferase reporter vector. The effect of TTP on nuclear hormone receptors transactivation was determined by assay of luciferase activity, as described under “Experimental Procedures.” Assays were performed in triplicate in three independent experiments in the presence (white bars) or absence (black bars) of the corresponding nuclear receptor ligands (17β-estradiol for ERα, progesterone for PR, 5α-Androstan-17β-ol-3-one for AR and dexamethasone for GR. The results are represented as mean ± S.E. Differences in ERα activity between control MCF-7 cells and TTP-expressing MCF-7 cells are statistically significant (p < 0.05).
dependent activation of GR has been associated to inhibition of the apoptotic response to chemotherapy treatment in breast cancer cells through its effect on the transcriptional activation of Bcl-xL, Bak, SGK-1 and MKP-1 [40–45]. Finally, different studies have suggested that crosstalk between AR and ERα is involved in the regulation of normal mammary gland and breast cancer development [46].

**Fig. 4.** siRNA-mediated knockdown of TTP protein levels in MCF-7 cells. MCF-7 cells were transfected with specific siRNA-TTP (1.25 μg) or with an unrelated control siRNA (2.5 μg). (A) Protein extracts from MCF-7 cultures were resolved by PAGE, and expression levels of TTP, ERα, PR, GR, AR and tubulin, as a loading control protein, were evaluated by Western blot using specific antibodies as described under “Experimental Procedures.” TTP knockdown increases ERα, PR, GR and AR transactivation. MCF-7 cells were transfected with an unrelated siRNA (control) or a specific TTP-siRNA (1.5 μg), along with TK-LUC, in the presence of the corresponding nuclear receptor ligands. The effect of TTP-siRNA on ERα (B), PR (C), GR (D) and AR (E) transactivation was determined by a luciferase assay and compared with luciferase activity in MCF-7 cells transfected with empty pCMV-3TAG vector and the corresponding Tk-LUC reporter vector. Results, in triplicate in three independent experiments, are represented as mean ± S.E. (error bars). Differences in ERα activity in MCF-7 cells transfected with TTP or with TTP-siRNA were shown to be statistically significant (p < 0.05).
breast cancer tumors, higher AR expression levels are associated with lower grade, reduced lymph node involvement and longer disease-free survival [47–50].

The function of TTP as a corepressor of multiple steroid nuclear receptors in MCF-7 cells suggest that this protein may participate in the down-regulation of the different functions of ERx, PR, GR and AR in mammary gland cells. The results from this work and previous studies that show that TTP expression is reduced in different types of cancer tumors supports the role of nuclear TTP as a tumor suppressor protein [19]. We propose that alterations in TTP expression may contribute to the development and progression of breast cancer tumors by the dysregulation of ERx and other steroid nuclear receptors signaling pathways. This hypothesis is supported by the observation that siRNA-mediated reduction of endogenous TTP expression levels produced a significant change in ERx, PR, GR and AR transactivation in MCF-7 cells. Based on the impact of the steroid nuclear receptors on the human genome, it is conceivable that TTP could participate in the transcriptional control of a numerous collection of genes. Further studies will be necessary to identify and characterize the genes regulated by nuclear TTP and to explore their potential as novel tumor markers or therapeutic targets for the diagnostic and treatment of breast cancer.

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