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PRECLINICAL STUDY

# Mammary differentiation induces expression of Tristetraprolin, a tumor suppressor AU-rich mRNA-binding protein

**M. Victoria Goddio · Albana Gattelli · Victoria Slomiansky · Ezequiel Lacunza · Timothy Gingerich · Johanna M. Tocci · María M. Facchinetti · Alejandro C. Curino · Jonathan LaMarre · Martín C. Abba · Edith C. Kordon**

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**Abstract** Tristetraprolin (TTP) is a RNA-binding protein that inhibits the expression of pro-inflammatory cytokines and invasiveness-associated genes. TTP levels are decreased in many different cancer types and it has been proposed that this protein could be used as a prognostic factor in breast cancer. Here, using publicly available DNA microarray datasets, "serial analysis of gene expression" libraries and qRT-PCR analysis, we determined that *TTP* mRNA is present in normal breast cells and its levels are significantly decreased in all breast cancer subtypes. In addition, by immunostaining, we found that TTP expression is higher in normal breast tissue and benign lesions than in infiltrating carcinomas. Among these, lower grade tumors showed increased TTP expression compared to higher grade cancers. Therefore, these data indicate that TTP protein levels would provide a better negative correlation with breast cancer invasiveness than TTP transcript levels. In mice, we found that *TTP* mRNA and protein expression is also diminished in mammary tumors. Interestingly, a strong positive association of TTP expression and mammary differentiation was identified in normal and tumor cells. In fact, TTP expression is highly increased during lactation, showing good correlation with various mammary differentiation factors. *TTP* expression was also induced in mammary HC11 cells treated with lactogenic hormones, mainly by prolactin, through Stat5A activation. The effect of this hormone was highly dependent on mammary differentiation status, as prolactin was unable to elicit a similar response in proliferating or neoplastic mammary cells. In summary, these studies show that TTP expression is strongly linked to the mammary differentiation program in human and mice, suggesting that this protein might play specific and relevant roles in the normal physiology of the gland.

**Keywords** Tristetraprolin · Breast cancer · Mammary gland · Lactation · Prolactin

M. V. Goddio · A. Gattelli · V. Slomiansky · J. M. Tocci · E. C. Kordon
LEGMA, IFIBYNE-CONICET, Departamento de Química Biológica, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Buenos Aires, Argentina

E. Lacunza · M. C. Abba
CINIBA, Facultad de Ciencias Médicas, Universidad Nacional de La Plata, La Plata, Argentina

T. Gingerich · J. LaMarre
OntarioVeterinarian College, University of Guelph, Ontario, Canada

M. M. Facchinetti · A. C. Curino
Laboratorio de Biología del Cáncer, INIBIBB-CONICET, Centro Científico Tecnológico, Bahía Blanca, Argentina

E. C. Kordon (✉)
Facultad de Ciencias Exactas y Naturales, Ciudad Universitaria, CM1, Pabellón 2, 2do piso, 1428 Ciudad Autónoma de Buenos Aires, Argentina
e-mail: ekordon@qb.fcen.uba.ar

## Introduction

It has been demonstrated that the stability of many mRNAs encoding oncoproteins, cytokines, and inflammatory mediators is controlled by AU-rich elements (AREs), sequences located within the 3′UTR of many transcripts [1, 2]. ARE-directed control of mRNA decay is mediated, in part, through interactions with specific ARE-binding proteins (AUBPs). One such protein is tristetraprolin (TTP), which accelerates the decay of targeted transcripts

[3]. It has been demonstrated that TTP reduces the expression of inflammatory cytokines and participates in glucocorticoid-mediated anti-inflammatory activity [4–6]. In addition, several reports indicate that TTP participates in the inhibition of tumor progression. *TTP* mRNA levels are significantly decreased in many tumor types, including breast cancer [7]. Furthermore, in human breast cell lines, TTP inhibits the expression of cancer-related genes [8, 9]. However, to date, factors or conditions that modulate TTP expression in the mammary gland have not been reported.

Mammary gland development is a complex, multistep process. From pregnancy to lactation, lobulo-alveolar growth is followed by the complete differentiation of mammary epithelium, which allows the production and secretion of milk proteins. During lactation, the transcription factor Stat5A and the glucocorticoid receptor (GR) synergize to induce milk protein expression [10]. At weaning, a rapid switch from survival to death signaling occurs, leading to involution, which involves extensive remodeling [11] and an innate immune response in the tissue [12]. Interestingly, involution-associated factors may be responsible for the poor prognosis of pregnancy-associated breast cancers [13].

When modeling mammary differentiation in culture, a correct microenvironment must be provided for the cells to respond optimally to lactogenic hormones. Co-cultivation of epithelial cells with fibroblasts or adipocytes, or cultivation of epithelial cells in the presence of extracellular matrix proteins is necessary [14, 15]. Mouse mammary HC11 cells are different, since they require neither co-cultivation nor addition of exogenous substrates (extracellular matrix proteins) to respond to the lactogenic hormones. HC11 cells display a normal phenotype [16] and can produce beta-casein when lactogenic hormones are added to confluent cells because they are able to produce their own extracellular matrix, which is important for their differentiation [17].

The present study was undertaken to characterize the TTP expression pattern in normal and tumor mammary cells, in culture and in vivo. We demonstrate that this protein appears important in mammary differentiation, suggesting that loss of TTP expression in breast cancer development might be associated to the impairment of the mammary cell fate program.

## Materials and methods

### Gene expression profiling in DNA microarray and SAGE databases

We analyzed publicly available breast cancer microarray data derived from diverse tumor types [18]. The gene expression matrix and the associated clinical data were obtained from the Rosetta Inpharmatics website (http://www.rii.com/publications/2002/nejm.html). Tumor classification, according to the five genomic intrinsic subtypes, was established based on reported data [19]. *TTP* expression was determined, and comparisons among subtypes were established by ANOVA.

Twenty nine SAGE (*serial analysis of gene expression*) libraries, described in [20], were combined with 17 SAGE libraries generated at the Polyak Laboratory, Dana-Farber Cancer Institute, Boston, MA, USA, which were downloaded from the Cancer Genome Anatomy Project, SAGE Genie database (http://cgap.nci.nih.gov/SAGE/). A total of 46 breast SAGE libraries were analyzed: 4 normal breast epithelium, 7 ductal carcinoma in situ, and 35 invasive ductal carcinomas. SAGE data management and tag-to-gene matching for *TTP* (ATGGTGGGGG) were performed with a suite of web-based SAGE library annotation tools that we have developed.

For in silico analysis of *TTP*, *PRL*, *PRLR*, *GR*, *STAT5,* and *STAT3* expression profiles, a mouse mammary gland study available at the gene expression omnibus database (GSE8191) [21] was used. This dataset includes 40 microarray samples from a FVB mouse mammary gland development series.

### Morphological and immunohistochemical studies

Breast tissue samples were obtained from Dr Leónidas Lucero Hospital (Bahía Blanca, Buenos Aires, Argentina) after approval by the institutional review board.

Human and mouse tissues were formalin-fixed, paraffin-embedded, and sectioned. Antigen retrieval was performed with 10 mM Citrate buffer pH 6.0. TTP antibody (sc-14030) (Santa Cruz Biotechnology) was used at dilutions ranging between 1:200 and 1:250. Immunodetection was performed with either the DakoCytomation LSAB + System-HRP (Dako) or the Vectastain Elite ABC immunoperoxidase system (Vector Laboratories). Sections were counter-stained with hematoxylin. For human breast cancer samples, staining intensity was graded as negative (−), weak (+), moderate (++), or strong (+++). The number of optical fields in a specimen that were positively stained was expressed as a percentage of the total number of optical fields in the slide. A sample was considered positive when more than 5 % of the breast epithelial cells were stained.

### Mouse mammary models

Balb/c mice were maintained in a pathogen-free, temperature-controlled environment on a 12/12 h light/dark cycle and given sterilized laboratory chow and water ad libitum. All animal studies were conducted in accordance with the NIH Guide for the Care and the Use of Laboratory

Animals. Mouse mammary tumors were obtained from FBN/V-TgN (MMTVPyVT) 634Mu1 (PymT) mice (The Jackson Laboratory) [22], from MMTV(LA) infected BALB/c mice [23] and from BALB/c mice inoculated with 4T1 cells, which are derived from MMTV(C3H) infected BALB/c mice [24]. To analyze TTP expression in vivo, mice were euthanized and #4 mammary glands and/or tumors were removed.

### Cell culture

HC11 cells were grown in RPMI 1640 (GIBCO) supplemented with 10 % fetal calf serum (FCS) and 5 μg/ml insulin (Sigma). Confluent HC11 cells were maintained in RPMI with 2 % FCS and 5 μg/ml insulin for 3 days after which 5 μg/ml ovine PRL (Sigma) and $10^{-7}$ M Dex (Sigma) were added. T47D cells were grown in DMEM (GIBCO) with 10 % FCS. Cells were left in serum-free media overnight before PRL (3 μg/ml) treatment. 4T1 cells were cultured in DMEM (Sigma) supplemented with 10 % FCS.

### Mouse TTP promoter plasmid

The reporter plasmid mTTP-LUC was constructed in two steps. A fragment of the mouse *TTP* gene from -1619 upstream of the transcription start site to the last base before the stop codon was amplified by PCR using genomic DNA isolated from Hepa1-6 cells using the following primers: 5′ATCacgcgtGAGACTGGAGGATCGCAAGTTC3′ and 5′AGTTgatatcCTATTTTTCGAACTGCGGGTGGCTCCACTCAGAGACAGAGATACGATTG3′. The underlined sequence was designed to encode a C-terminal, 8 amino acid strep-tag, and a stop codon. The PCR product was digested with MluI and EcoRV (restriction site sequences were engineered into the primers and are shown in lower case). The plasmid pcDNA3.1(−) (Invitrogen) was digested with the same enzymes to remove the existing CMV promoter, 5′UTR, and a portion of the multiple cloning site. This fragment was then ligated to the PCR product to create pTTP-Gene-Flawed1. A fragment containing 1,561 bp of the *TTP* promoter and 31 bp of the *TTP* 5′UTR was excised from pTTP-Gene-Flawed1 using BglII and NcoI. pGL3-Basic was digested with the same enzymes and ligated to the TTP promoter fragment to create mTTP-LUC. Construction of pStat5A-WT and pSat5A-DN has been previously described [25, 26].

### Transfections

Using PEI reagent (Polysciences), HC11 cells were transfected with 1 μg mTTP-LUC, or pGL3-Basic, and 1 μg of the β-galactosidase-expressing plasmid pCMV-LacZ, as a control for transfection efficiency. Competent cells were treated with PRL (5 μg/ml) and Dex ($10^{-7}$M) for 24 h in serum-free media. To study Stat5A, cells were co-transfected with 1 μg mTTP-LUC or pGL3-Basic, 1 μg pStat5A-WT or pSat5A-DN, and pCMV-LacZ.

### Real-time quantitative RT-PCR (qRT-PCR) and western blot analyses

RNA was isolated using Trizol (Invitrogen) and reverse transcribed using a superscript™ II reverse transcriptase kit (Invitrogen). Real-time PCR reactions were performed in a Stratagene MX30005P thermal cycler (Agilent) using PerfeCta SYBR Green SuperMix (Quanta Biosciences). Primers used were: human TTP: 5′CATGGCCAACCGTTACACC3′ and 5′ACTCAGTCCCTCCATGGTCG3′, mouse TTP: 5′CGGAACTCTGCCACAAG3′ and 5′GGCGAAAAGGAACAAGA3′, mouse beta casein: 5′TCCCACAAAACATCCAGCC3′ and 5′ACGGAATGTT-GTGGAGTGG3′, mouse beta actin: 5′-CGGTTGGCCTTAGGGTTCAGGGGGGG-3′ and 5′-GTGGGCCGCTCTAGGCACCA-3′ and human beta actin: CGGTTG-GCCTTAGGGTTCAGGGGGG-3′ and 5′-GTGGGCCGCTCTAGGCAC-3′. Samples were run in triplicate and normalized to beta-actin. Values were calculated by the efficiency-corrected comparative $\Delta\Delta Ct$ method. Means and standard errors from at least 3 experiments were calculated and expressed as fold changes relative to controls.

Western blotting was performed as described [27]. Blots were probed for TTP and beta-actin with antibodies from Santa Cruz Biotechnology.

### Statistical analysis

Graphical data points are shown as the mean ± standard error. Statistical analysis was performed by means of STATISTICA 6.0 (StatSoft). Differences were regarded as significant at $p < 0.05$.

## Results

### TTP expression analysis in human breast samples

To explore the *TTP* mRNA expression pattern in breast cancer, we analyzed DNA microarray datasets of 295 primary invasive breast carcinomas classified according to the five genomic intrinsic subtypes [18]. Figure 1a shows that *Normal-like* primary breast carcinomas expressed higher *TTP* mRNA levels than the other breast cancer subtypes while no significant differences were found among the *Luminal-A*, *HER2+/ER-*, and *Basal-like* tumors. In contrast, quantitative RT-PCR analysis showed higher *TTP*

mRNA expression in the luminal tumor-derived cell lines MCF7, T47D, and ZR75-1 (poorly invasive and epithelioid) compared to the basal-like MDA-MB-231 cell line (highly invasive and fibroblastoid) (Fig. 1b).

To extend our analysis to non-tumorigenic breast samples, we evaluated a dataset containing 46 normal and breast cancer SAGE libraries [20]. Figure 1c shows that *TTP* mRNA is highly expressed in normal breast epithelium compared to carcinomas ($p < 0.0001$). However, no differences were found between in situ and invasive tumors. These observations were further validated in an independent set of human breast tissue samples by qRT-PCR. In agreement with our SAGE analysis, we found that normal mammary glands displayed higher expression than invasive carcinomas ($p = 0.017$). Interestingly, *TTP* expression was also lower in normal epithelium adjacent to tumors (Fig. 1d).

TTP protein expression was analyzed in 64 breast tissue sections by immunohistochemistry (IHC). Left panels of Fig. 2 show that normal breast tissue (A, B) and benign lesions (atypical hyperplasia, C), displayed positive staining in epithelial cells, while most undifferentiated invasive carcinomas did not have detectable TTP expression (D; 10×). Samples were divided into two groups according to their histopathologic diagnosis: normal breast epithelium and benign lesions ($n = 17$) and infiltrating ductal carcinomas ($n = 47$), which were also subdivided according to the histologic tumor grade (Fig. 2 right panels, E&F). TTP protein expression was detected in 82 % (14 out of 17) of the normal/benign samples analyzed, most of which (12 out of 17) showed moderate to strong intensity of staining. A statistically significant decrease in staining intensity was observed when the number of normal-benign lesions with moderate to strong TTP staining was compared to the proportion of infiltrating carcinomas demonstrating the same level of staining ($p = 0.02$). In contrast, 63 % (31 out of 47) of the invasive ductal carcinomas showed negative or weak TTP expression. In these samples, decreased TTP expression correlated with tumor grade ($p = 0.01$, Fig. 2f). The well-differentiated carcinomas showed moderate to strong TTP expression, while the undifferentiated carcinomas were mostly negative for TTP protein expression. Interestingly, no statistically significant association ($p > 0.05$) was detected between TTP expression and the ER-alpha status determined by IHC in these samples (data not shown).

## TTP regulation in mouse mammary cells

In mouse tissue, IHC analysis also revealed that undifferentiated mammary tumors derived from either PymT (Fig. 3a), 4T1 cells or MMTV infection, showed very weak TTP staining compared to more differentiated carcinomas (MMTV-induced, Fig. 3b) or non-neoplastic mammary tissue (Fig. 3c). Interestingly, in the mammary glands of healthy BALB/c mice, we found that lactating females showed stronger epithelial expression than either virgin or involuting glands (Figs 3d–f).

In agreement with the pattern observed by IHC, qRT-PCR analysis also showed that *TTP* mRNA expression was much higher in differentiated non-neoplastic mammary cells (prolactin-treated HC11) than in mammary tumor cells (Fig. 4a). In addition, we determined that *TTP* mRNA is strongly induced during lactation in BALB/c female mice (Fig. 4b). A publicly available mouse mammary gland gene expression dataset from FVB mice also showed that *TTP* mRNA peaks at the 9th day of lactation (Fig. 4c) and co-expression analysis demonstrated significant

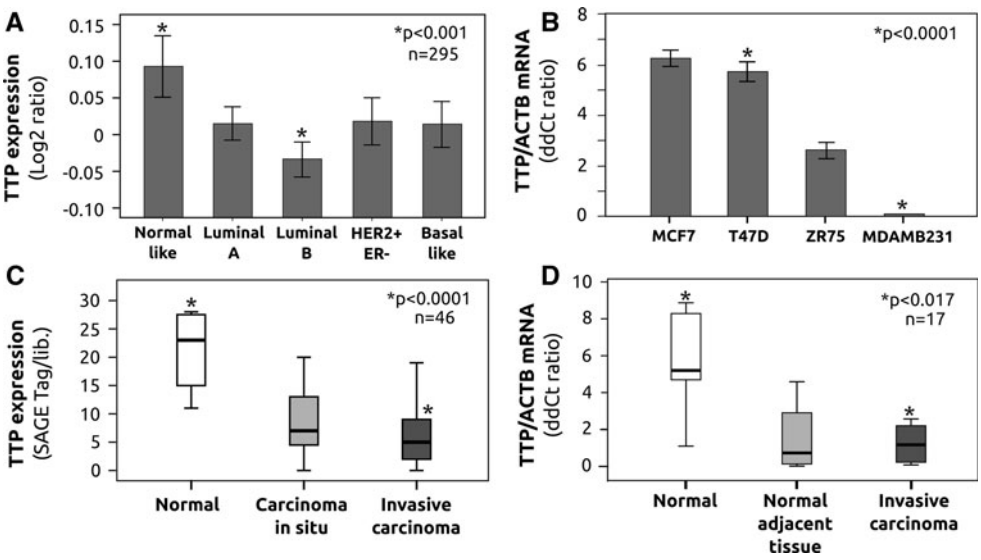


**Fig. 1** *TTP* mRNA expression in human breast. *TTP* expression profile obtained from publicly available DNA microarray datasets classified according to the genomic intrinsic subtypes (**a**); *TTP* expression in MCF7, T47D, ZR75-1, and MDA-MB 231 breast cancer cell lines (**b**); serial analysis of gene expression of *TTP* tag in breast SAGE libraries (**c**); *TTP* mRNA expression normalized to beta-actin as determined by qRT-PCR in breast samples (**d**)

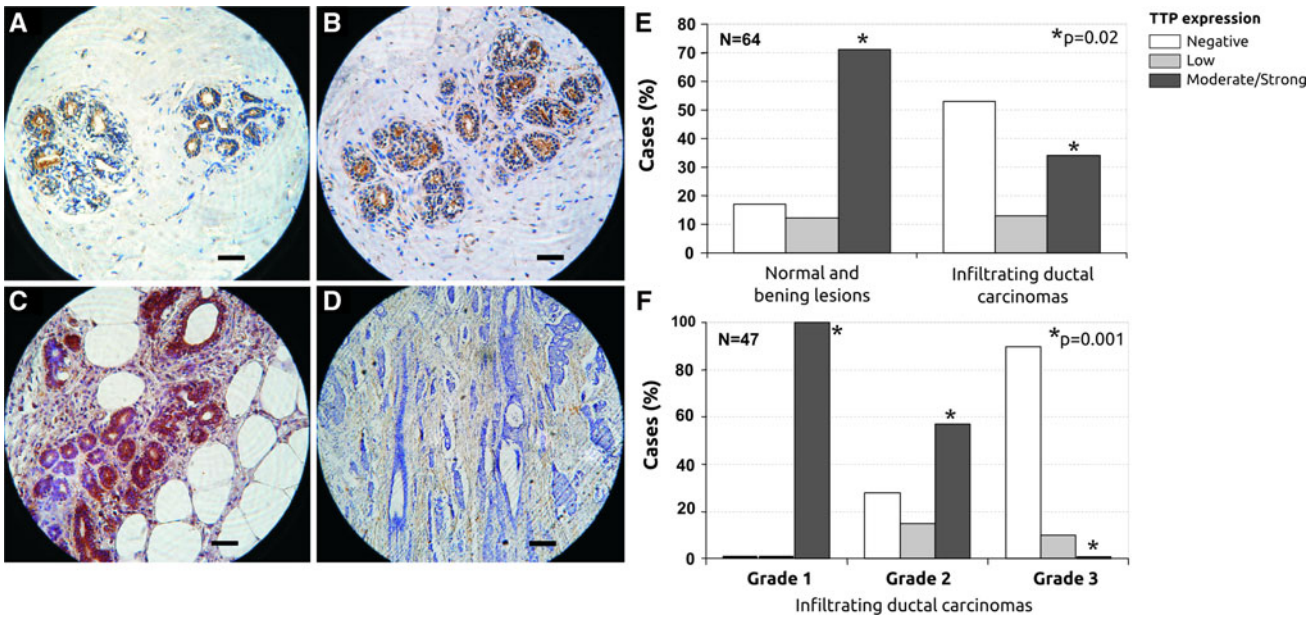


**Fig. 2** TTP protein expression in human breast samples. Immunohistochemical (IHC) staining for TTP protein in normal breast tissue (**a**, **b**; 40×), benign lesion (atypical hyperplasia) (**c**; 40×), and undifferentiated invasive carcinoma (**d**; 10×). Strong expression of TTP with a predominant cytoplasmic staining is observed in the normal and hyperplasic tissues. *Scale bars* represent 10 μm in **a**, **b**, **c**, and 40 μm in **d**. Distribution of total cases ($n = 64$) divided into normal and benign lesions ($n = 17$) and tumor samples (Infiltrating ductal carcinomas, $n = 47$) according to TTP expression levels (**e**). Distribution of the Infiltrating ductal carcinomas according to TTP expression levels and histologic tumor grade (**f**)

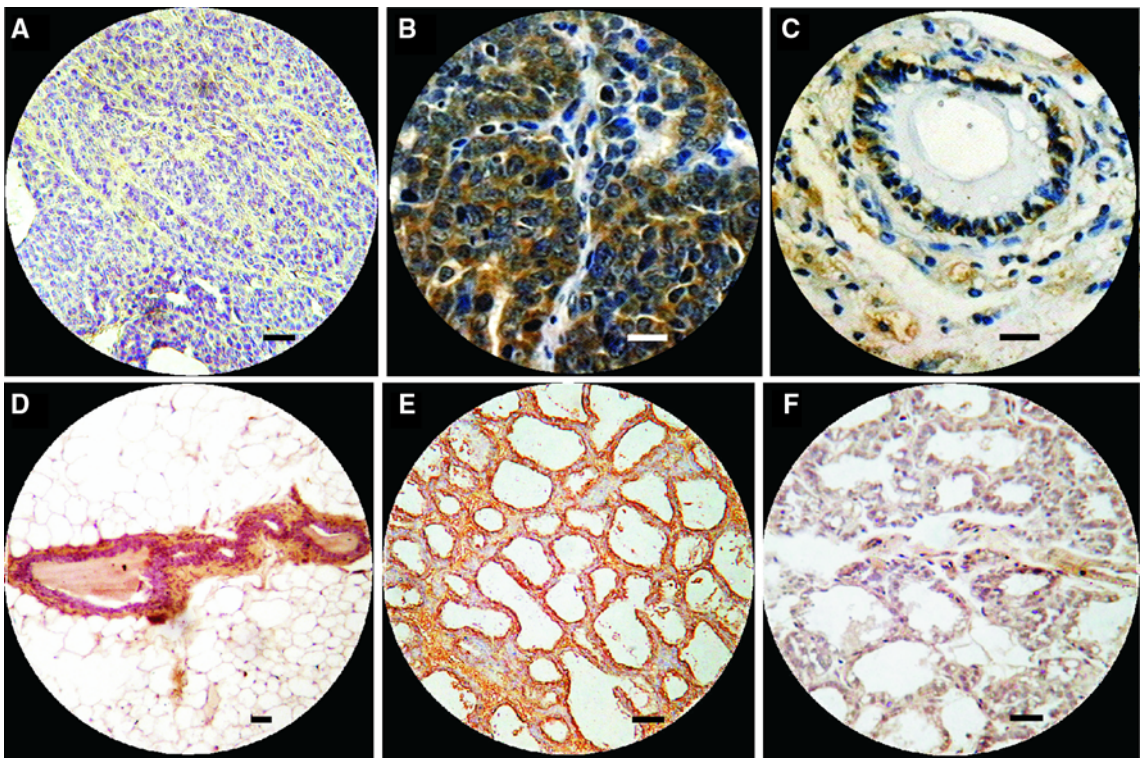


**Fig. 3** TTP protein expression in mouse mammary gland samples. IHC analysis in undifferentiated mammary tumor (**a**; 40×), a section of tissue derived from a differentiated tumor (**b**; 60×), normal mammary gland (**c**; 40×), mouse mammary gland from a virgin female (**d**; 10×), lactating gland (**e**; 40×) and 48 h involuting mammary tissue (**f**; 40×). Strong expression of TTP with cytoplasmic staining is observed in the normal and the differentiated mammary tumor. *Scale bars* represent 10 μm in **a**, **b**, **c**, **e**, **f**, and 40 μm in **d**

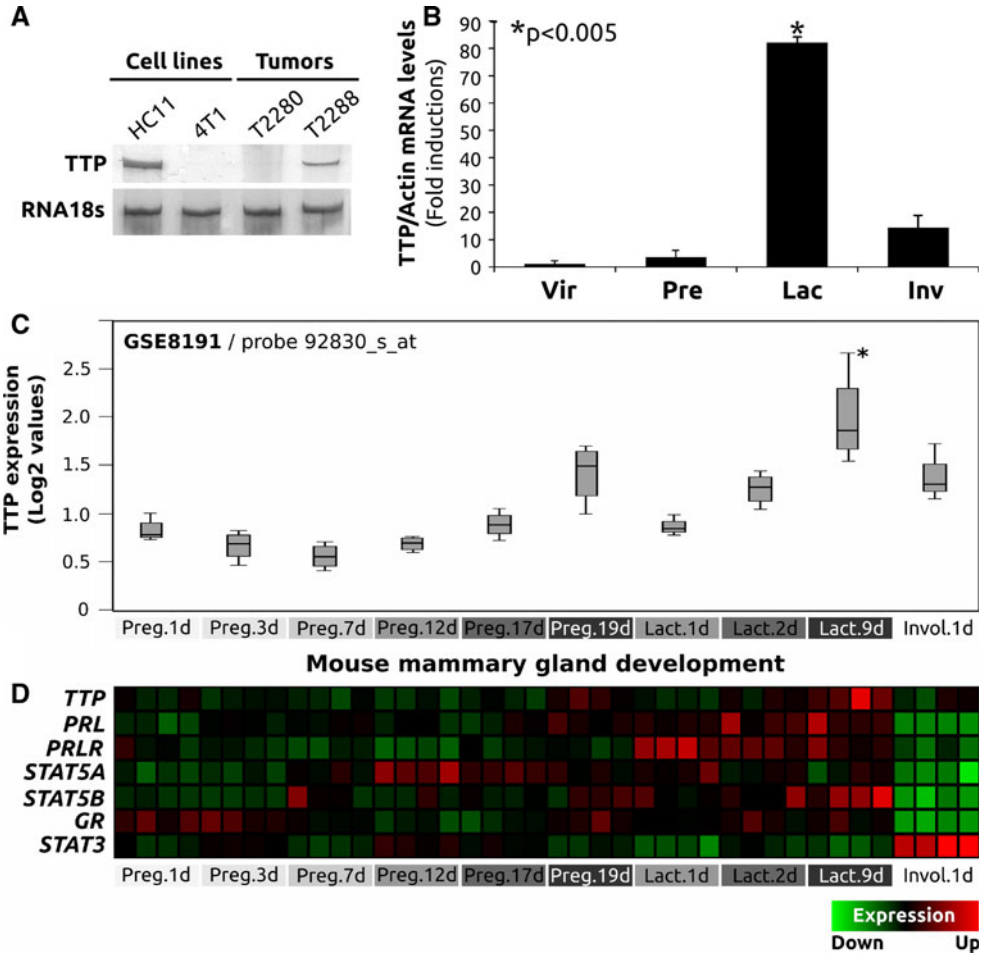


**Fig. 4** *TTP* mRNA expression during mouse mammary gland development and tumor samples. RT-PCR analysis of *TTP* expression in normal and cancer mammary cell lines (HC11 and 4T1, respectively), undifferentiated (T2280), and well-differentiated (T2288) mouse mammary tumors (**a**). *TTP* mRNA expression in virgin (Vir), pregnant (Pre), lactating (Lac) and 24 h post-weaning (Inv) BALB/C mice, * $p < 0.00005$ (**b**). Box plots of *TTP* expression levels (Affymetrix probe ID 92830_s_at) during pregnancy, lactation, and involution of FVB mouse mammary glands based on a publicly available gene expression dataset (GSE8191) (**c**). Analysis of *TTP* co-expression with *PRL*, *PRLR*, *GR*, *STAT5*, and *STAT3* mRNAs. Each one of the 10 time points was analyzed by performing four biologic replicates (40 microarray samples) (**d**)

positive correlations with mRNAs encoding proteins that play relevant roles in mammary differentiation such as prolactin (*PRL*, $r = 0.614$; $p < 0.0001$), prolactin-receptor (*PRLR*, $r = 0.683$; $p < 0.0001$), and *STAT5B* ($r = 0.697$; $p < 0.0001$) (Fig. 4d).

To further define the association between mammary differentiation and *TTP* expression, the non-tumorigenic HC11 mouse mammary cell line was used. In order to respond to lactogenic hormones (i.e., to become "competent"), these cells must remain 100 % confluent for at least 2 days [17]. Our results show that *TTP* expression was induced in competent HC11 cells and its mRNA levels were further increased by the addition of PRL and dexamethasone (Dex) (Fig. 5a). Treating with PRL alone for 6 h also induced TTP expression although to a lesser extent than both hormones together (Fig. 5b). Alternatively, Dex treatment alone induced TTP expression although the response was different depending on whether the hormone was added to proliferating or competent cells. Proliferating cells responded in a dose-dependent manner (Fig. 5c), while in competent cells the effect was milder and the greatest response was observed at 10 nM, which corresponds to the concentration used to induce milk protein expression (Fig. 5d).

To determine whether the effect of lactogenic hormones on competent HC11 cells was mediated by promoter activation, subconfluent cells were transfected with the mTTP-LUC plasmid. After reaching competency, cells were treated with hormones for 24 h. We found that PRL induced luciferase activity with or without Dex, while the glucocorticoid alone did not exert any effect. Importantly, co-transfection with a Stat5A dominant-negative mutant (Stat5A-DN) completely blocked the induction of TTP-driven luciferase activity by PRL (Fig. 6a). The relevance of stable, lactogenesis-associated, Stat5A activation for *TTP* induction in mammary cells was also indicated by the lack of response to PRL treatment in proliferating HC11 cells (data not shown). Interestingly, PRL treatment in competent HC11 cells required a minimum of 6 h to induce a significant *TTP* mRNA increase (Fig. 6b), while, in T47D cells, the effects were observed starting at 15 min, reaching a peak at 1 h and declining rapidly to return to control levels by 2 h (Fig. 6c). However, PRL-driven *TTP* induction in T47D cells was not blocked by AG490 co-treatment (data not shown), suggesting that JAK2/Stat5A pathway activation was not mediating short-term *TTP* induction in breast cancer cells. Figure 6d shows that, upon PRL

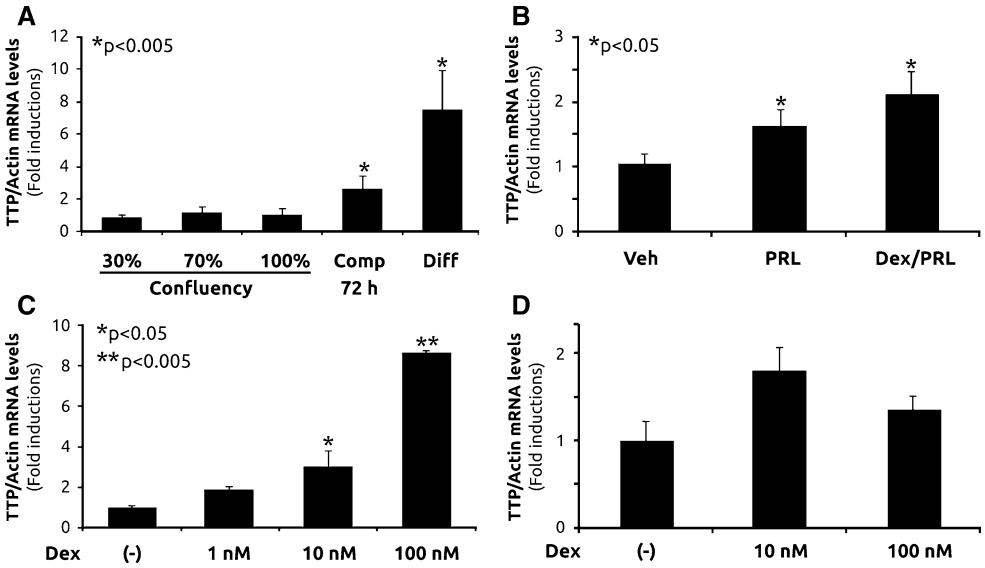


**Fig. 5** *TTP* mRNA expression in HC11 mouse mammary cells. *TTP* expression in HC11 cells that are: subconfluent (30–70 %), confluent (100 %), competent (Comp 72 h), and differentiated (Diff) (**a**). *TTP* expression in competent HC11 cells treated with PRL or Dex and PRL (Dex/PRL) for 6 h (**b**). Effect of Dex at different doses, for 8 h, on TTP levels in proliferating (**c**) and competent (**d**) HC11 cells

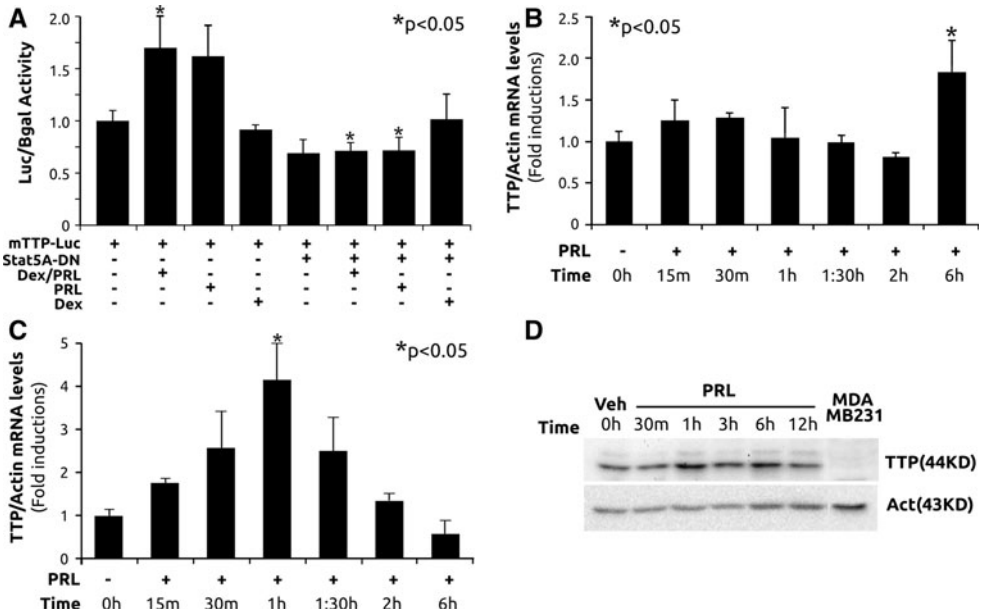


**Fig. 6** Effect of lactogenic hormones on *TTP* expression. Subconfluent HC11 cells were transfected with the mTTP-LUC construct with or without Stat5A-DN plasmid. After cells became competent, luciferase activity was measured upon treatment with prolactin (PRL), Dexamethasone (Dex), both hormones (Dex/PRL) or vehicle (Veh) for 24 h in serum-free media (**a**). *TTP* mRNA expression in competent HC11 (**b**) and subconfluent T47D cells (**c**) treated with prolactin (PRL) for different times. Representative western blot assay showing TTP protein expression in T47D cells treated with PRL for different times; last lane shows untreated MDA-MB231 cells used as negative control for TTP expression (**d**)

treatment, TTP protein expression was also rapidly augmented and declined by 12 h in these breast cancer cells.

## Discussion

Several recent reports have shown that decreased TTP expression is a strong indicator of poor prognosis for breast cancer patients. However, the significance of evaluating *TTP* mRNA is under debate since Griseri et al. [8] have indicated that the level of TTP protein, but not *TTP* mRNA, is a key factor to predict tumor aggressive behavior. Our results appear to support this concept. The analysis of a microarray dataset derived from tumors classified according to five genomic intrinsic subtypes shows that high *TTP* mRNA expression is most highly associated with *Normal-like* breast carcinomas. This cancer subtype is characterized by relatively high expression of many genes expressed

in normal breast epithelium; however, survival times and relapse-free survival are shorter in this category when compared to *Luminal A*. In addition, *Luminal A* and *Basal* tumors showed similar *TTP* mRNA levels although the prognosis of these cancer subtypes are dramatically different [28, 29].

Analysis of SAGE libraries, which included normal breast samples, showed a negative correlation between *TTP* mRNA levels and cancer phenotype. However, once again, there were no differences between in situ and invasive carcinomas. In addition, qRT-PCR analysis of samples from normal mammary tissue adjacent to carcinoma showed inhibition of *TTP* expression, suggesting that lower *TTP* mRNA levels are associated with cancer development but not necessarily with cancer invasiveness. Nevertheless, Brennan et al. [7] showed a significantly negative correlation between *Tumor grade* and *TTP* mRNA levels. Since *Tumor grading* only classifies invasive carcinomas, while other types of breast lesions are not included in these categorizations [30], it is possible that, among invasive carcinomas, less aggressive *Grade 1* tumors express higher *TTP* mRNA levels than the more aggressive *Grade 3* lesions. Similarly, using the *Intrinsic phenotype* classification, we found that *Luminal B* tumors, (which show worse outcomes than those classified as *Luminal A* [28]), expressed lower amounts of *TTP* mRNA. Therefore, we conclude that the normal breast gene expression profile would include higher *TTP* mRNA levels than any tumor subtype. In addition, more aggressive breast cancer subtypes may have reduced TTP translation efficiency, as previously reported for certain TTP allelic variants [8]. Therefore, in those cases, protein expression would be lower at similar mRNA levels. Finally, in specific tumor subtypes, with similar translation efficiency, aggressiveness may negatively correlate with individual *TTP* mRNA levels.

The results of our IHC analysis confirm that TTP protein levels represent useful elements of breast cancer diagnosis and analysis. To the best of our knowledge, this is the first report that shows a negative correlation between TTP expression and tumor invasiveness by this technique, which is the most commonly employed in breast cancer diagnosis. Therefore, our results increase the potential utility of using TTP protein as a prognostic factor for this disease.

The conspicuous presence of TTP protein and mRNA in the normal breast epithelium and its inhibition in breast cancer cells suggested to us that expression of this protein might be modulated by mammary-specific factors. To test this hypothesis, we analyzed mouse mammary glands at different developmental stages and we found that TTP is induced during lactogenesis.

Glucocorticoids and PRL induced lactogenic differentiation, and glucocorticoids are recognized to increase TTP expression in lung epithelial cells [5]. Interestingly, we found that Dex produced a similar effect in proliferating HC11 cells; however, when mammary cells were allowed to develop the lactogenic program, PRL became more important than glucocorticoids for stable TTP induction.

PRL induces terminal differentiation of mammary epithelial cells and synthesis of milk components during lactation through the Jak2-Stat5 pathway [31, 32]. We found that PRL, with or without Dex, induced mouse promoter activation in competent HC11 cells, and LUC activity was completely blocked by the addition of a Stat5A dominant-negative mutant. Therefore, at least part of PRL effect on endogenous *TTP* expression in differentiated mouse mammary cells is likely due to the interaction of Stat5A with mouse TTP promoter sequences. Curiously, in mast cells, Stat5 mediates *TTP* repression [33], which contrasts the ability of Stat6 and Stat3 to induce its expression [34, 35]. Therefore, Stat factors might recruit either transcriptional repressors or activators to the *TTP* promoter, depending on the cell type and/or physiologic status, which would allow the generation of the appropriate cell responses to specific tissue conditions.

The capacity of PRL to induce stable, long-term *TTP* expression in mammary cells seems to be essentially associated with lactogenic differentiation. PRL did not induce *TTP* transcription in proliferating HC11 cells; in T47D, it occurred only transiently and independent of the JAK2-Stat5A pathway. The rapid induction observed in this cancer cell line is consistent with previous reports that described TTP as the product of an immediate-early response gene (*ZFP-36*) that is rapidly induced in various cell types by a number of different stimuli, each reflecting a transient, immediate-early response after which *TTP* mRNA returns to basal or near-basal levels [36–39]. Therefore, we conclude that in the normal mammary epithelium, *TTP* expression is specifically and differentially regulated.

In summary, our results indicate that TTP is highly expressed in the normal mammary epithelium, while its downregulation frequently accompanies tumorigenesis. We also observed that, at least in the mouse gland, lactogenesis enhances TTP expression, probably through Stat5A activation. We believe that these studies reveal a new potential biologic role for this tumor suppressor protein in mammary epithelium, since TTP might protect the tissue from inflammatory and/or remodeling activities that would trigger involution of the gland. In our laboratory, experiments are underway to explore this possibility.

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**Ethical standards** All the experiments described herein comply with the current laws of Argentina, where they were performed and conform to the guidelines of the Canadian Council on Animal Care.

**Conflict of interests** The authors declare no conflict of interest for the studies described.

## References


1. Chen CYA, Shyu AB (1995) AU-rich elements: characterization and importance in mRNA degradation. Trends Biochem Sci 20: 465–470
2. Guhaniyogi J, Brewer G (2001) Regulation of mRNA stability in mammalian cells. Gene 265:11–23
3. Barreau C, Paillard L, Osborne HB (2005) AU-rich elements and associated factors: are there unifying principles? Nucleic Acids Res 33:7138–7150
4. Taylor GA, Carballo E, Lee DM, Lai WS, Thompson MJ, Patel D, Schenkman DI, Gilkeson GS, Broxmeyer HE, Haynes BF, Blackshear PJ (1996) A pathogenetic role for TNF alpha in the syndrome of cachexia, arthritis, and autoimmunity resulting from tristetraprolin (TTP) deficiency. Immunity 4:445–454
5. Smoak K, Cidlowski JA (2006) Glucocorticoids regulate tristetraprolin synthesis and posttranscriptionally regulate tumor necrosis factor alpha inflammatory signaling. Mol Cell Biol 26:9126–9135
6. Ishmael FT, Fang X, Galdiero MR, Atasoy U, Rigby WF, Gorospe M, Cheadle C, Stellato C (2008) Role of the RNA-binding protein tristetraprolin in glucocorticoid-mediated gene regulation. J Immunol 180:8342–8353
7. Brennan S, Kuwano Y, Alkharouf N, Blackshear PJ, Gorospe M, Wilson GM (2009) The mRNA-destabilizing protein tristetraprolin is suppressed in many cancers, altering tumorigenic phenotypes and patient prognosis. Cancer Res 69:5168–5176
8. Griseri P, Bourcier C, Hieblot C, Essafi-Benkhadir K, Chamorey E, Touriol C, Pagès G (2011) A synonymous polymorphism of the Tristetraprolin (TTP) gene, an AU-rich mRNA-binding protein, affects translation efficiency and response to Herceptin treatment in breast cancer patients. Hum Mol Genet 20: 4556–4568
9. Al-Souhibani N, Al-Ahmadi W, Hesketh JE, Blackshear PJ, Khabar KSA (2010) The RNA-binding zinc-finger protein tristetraprolin regulates AU-rich mRNAs involved in breast cancer-related processes. Oncogene 29:1–11
10. Reichardt HM, Horsch K, Grone HJ, Kolbus A, Beug H, Hynes N, Schütz G (2001) Mammary gland development and lactation are controlled by different glucocorticoid receptor activities. Eur J Endocrinol 145:519–527
11. Strange R, Li F, Saurer S, Burkhardt A, Friis RR (1992) Apoptotic cell death and tissue remodelling during mouse mammary gland involution. Development 115:49–58
12. Pensa S, Watson CJ, Poli V (2009) Stat3 and the inflammation/acute phase response in involution and breast cancer. J Mammary Gland Biol Neoplasia 14:121–129
13. Lyons TR, Schedin PJ, Borges VF (2009) Pregnancy and breast cancer: when they collide. J Mammary Gland Biol Neoplasia 14:87–98
14. Streuli CH (1993) Extracellular matrix and gene expression in mammary epithelium. Semin Cell Biol 4:203–212
15. Lin CQ, Bissell MJ (1993) Multi-faceted regulation of cell differentiation by extracellular matrix. FASEB J 7:737–743
16. Danielson KG, Oborn CJ, Durban EM, Butel JS, Medina D (1984) Epithelial mouse mammary cell line exhibiting normal morphogenesis in vivo and functional differentiation in vitro. Proc Natl Acad Sci USA 81:3756–3760
17. Chammas R, Taverna D, Cella N, Santos C, Hynes NE (1994) Laminin and tenascin assembly and expression regulate HC11 mouse mammary cell differentiation. J Cell Sci 107:1031–1040
18. van de Vijver MJ, He YD, Van't Veer LJ, Dai H, Hart AAM, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D et al (2002) A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 347: 1999–2009
19. Fan C, Oh DS, Wessels L, Weigelt B, Nuyten DS, Nobel AB, van't Veer LJ, Perou CM (2006) Concordance among gene-expression-based predictors for breast cancer. N Engl J Med 355:560–569
20. Abba MC, Sun H, Hawkins KA, Drake JA, Hu Y, Nunez MI, Gaddis S, Shi T, Horvath S, Sahin A, Aldaz CM (2007) Breast cancer molecular signatures as determined by SAGE: correlation with lymph node status. Mol Cancer Res 5:881–890
21. Anderson SM, Rudolph MC, McManaman JL, Neville MC (2007) Key stages in mammary gland development. Secretory activation in the mammary gland: it's not just about milk protein synthesis! Breast Cancer Res 9:204
22. Curino AC, Engelholm LH, Yamada SS, Holmbeck K, Lund RL, Molinolo AA, Behrendt N, Boye SN, Bugge TH (2005) Intracellular collagen degradation mediated by uPARAP/Endo180 is a major pathway of extracellular matrix turnover during malignancy. J Cell Biol 169(6):977–985
23. Buggiano V, Schere-Levy C, Gattelli A, Cirio MC, Marfil M, Nepomnaschy I, Piazzon I, Helguero L, Vanzulli S, Kordon E (2002) Origin and progression of pregnancy-dependent mammary tumors induced by new mouse mammary tumor virus variants. Breast Can Res Treat 75:191–202
24. Dexter DL, Kowalski HM, Blazar BA, Fligiel Z, Vogel R, Heppner GH (1978) Heterogeneity of tumor cells from a single mouse mammary tumor. Cancer Res 38:3174–3181
25. Beuvink I, Hess D, Flotow H, Hofsteenge J, Groner B, Hynes NE (2000) Stat5a serine phosphorylation. Serine 779 is constitutively phosphorylated in the mammary gland, and serine 725 phosphorylation influences prolactin-stimulated in vitro DNA binding activity. J Biol Chem 275:10247–10255
26. Moriggl R, Gouilleux-Gruart V, Jahne R, Berchtold S, Gartmann C, Liu X, Hennighausen L, Sotiropoulos A, Groner B, Gouilleux F (1996) Deletion of the carboxyl-terminal transactivation domain of MGF-Stat5 results in sustained DNA binding and a dominant negative phenotype. Mol Cell Biol 16:5691–5700
27. Bertucci PY, Quaglino A, Pozzi AG, Kordon EC, Pecci A (2010) Glucocorticoid-induced impairment of mammary gland involution is associated with STAT5 and STAT3 signaling modulation. Endocrinology 151:5730–5740
28. Sørlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H et al (2001) Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci USA 98:10869–10874
29. Lonning PE, Sorlie T, Perou CM, Brown PO, Botstein D, Borresen-Dale A-L (2001) Microarrays in primary breast cancer—lessons from chemotherapy studies. Endocr Relat Cancer 8: 259–263
30. Elston CW, Ellis IO (1991) Pathological prognostic factors in breast cancer. I. The value of histological grade in breast cancer: experience from a large study with long-term follow-up. Histopathology 19:403–410

31. Ormandy CJ, Naylor M, Harris J, Robertson F, Horseman ND, Lindeman GJ (2003) Investigation of the transcriptional changes underlying functional defects in the mammary glands of prolactin receptor knockout mice. Recent Prog Horm Res 58:297–323
32. Watson CJ, Burdon TG (1996) Prolactin signal transduction mechanisms in the mammary gland: the role of the Jak/Stat pathway. Rev Reprod 1:1–5
33. Barnstein BO, Li G, Wang Z, Kennedy S, Chalfant C, Nakajima H, Bunting KD, Ryan JJ (2006) Stat5 expression is required for IgE-mediated mast cell function. J Immunology 177:3421–3426
34. Suzuki K, Nakajima H, Ikeda K, Maezawa Y, Suto A, Takatori H, Saito Y, Iwamoto I (2003) IL-4-Stat6 signaling induces tristetraprolin expression and inhibits TNF-alpha production in mast cells. J Exp Med 198:1717–1727
35. Joe Y, Kim HJ, Kim S, Chung J, Ko MS, Lee WH, Chang KC, Park JW, Chung HT (2011) Tristetraprolin mediates anti-inflammatory effects of nicotine in lipopolysaccharide-stimulated macrophages. J Biol Chem 286:24735–24742
36. DuBois RN, McLane MW, Ryder K, Lau LF, Nathans D (1990) A growth factor-inducible nuclear protein with a novel cysteine/histidine repetitive sequence. J Biol Chem 265:19185–19191
37. Lai WS, Stumpo DJ, Blackshear PJ (1990) Rapid insulin-stimulated accumulation of an mRNA encoding a proline-rich protein. J Biol Chem 265:16556–16563
38. Mittelstadt PR, DeFranco A (2003) Induction of early response genes by cross-linking membrane Ig on B lymphocytes. J Immunol 150:4822–4832
39. Ogawa K, Chen F, Kim YJ, Chen Y (2003) Transcriptional regulation of tristetraprolin by transforming growth factor-beta in human T cells. J Biol Chem 278:30373–30381