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Hypothyroidism decreases JAK/STAT signaling pathway in lactating rat mammary gland

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ABSTRACT

Thyroid pathologies have deleterious effects on lactation. Especially hypothyroidism (HypoT) induces premature mammary involution at the end of lactation and decreases milk production and quality in mid lactation. Milk synthesis is controlled by JAK2/STAT5 signaling pathway and prolactin (PRL), which activates the pathway. In this work we analyzed the effect of chronic 6-propyl-2-thiouracil (PTU)-induced HypoT on PRL signaling pathway on mammary glands from rats on lactation (L) days 2, 7 and 14. HypoT decreased prolactin receptor expression, and expression and activation of Stat5a/b protein. Expression of members of the SOCS-CIS family, inhibitors of the JAK-STAT pathway, decreased in L2 and L7, possibly as a compensatory response of the mammary cells to maintain PRL responsiveness. However, on L14, the level of these inhibitors was normal and the transcription of α-lactoalbumin (*lalba*), a target gene of the PRL pathway, decreased by half. HypoT altered the transcriptional capacity of the cell and decreased mRNA levels of Prlr and *Stat5b* on L14. *Stat5b* gene has functional thyroid hormone response elements in the regulatory regions, that bind thyroid hormone receptor β (TRβ) differentially and in a thyroid hormone dependent manner. The overall decrease in the PRL signaling pathway and consequently in target gene (*lalba*) mRNA transcription explain the profound negative impact of HypoT on mammary function through lactation.

1. Introduction

Thyroid hormones (THs) in physiological concentrations are essential for prolactin (PRL) actions in the mammary gland (MG) (Campo Verde Arbocco et al., 2016; Campo Verde Arbocco et al., 2015; Pergialiotis et al., 2015; Hapon et al., 2007a; Varas and Jahn, 2005; Varas et al., 2001, 2002). Small doses of THs administered during lactation to otherwise euthyroid cows increase milk production proving a direct TH action in the regulation of lactation (Capuco and Akers, 1999; Capuco et al., 1999, 2008). However, exposure to excessive or insufficient quantities of THs during lactation diminishes milk production and quality and advances mammary involution (Campo Verde Arbocco et al., 2016; Varas et al., 2002). In fact, mammary expression of deiodinase type I, that converts tetraiodothyronine (T4) to triiodothyronine (T3), decreases in the peripartum period, most possibly to protect the MG from the deleterious actions of excessive THs (Anguiano et al., 2004).

Thyroid diseases are quite common in women during pregnancy and lactation. The deleterious effect of these pathologies in female reproduction has been amply described by our workgroup. Chronic hyperthyroidism (HyperT) in rats advances luteolysis (Navas et al., 2011) and delivery (Rosato et al., 1992, 1998), has adverse effects on maternal behavior and milk ejection (Varas and Jahn, 2005; Rosato et al., 1992) and disrupts MG lipid metabolism in lactating rats (Varas et al., 2001). We have also shown that hypothyroidism (HypoT) is associated with decreased litter growth and milk quality, abnormal hormonal levels and hormone receptors expression, and decreased milk ejection in response to suckling (Campo Verde

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Arbocco et al., 2015; Hapon et al., 2003, 2007a). One of the most striking effects of both thyroid pathologies is the induction of premature mammary involution at the end of lactation associated with milk stasis (Campo Verde Arbocco et al., 2016; Varas et al., 2002). There is evidence showing that the presence of adequate concentrations of THs is essential for milk production in response to PRL (Capuco and Akers, 1999; Capuco et al., 2008). In concordance with this, the premature mammary involution that we observed in lactating hypothyroid rats is associated with a disruption in PRL signaling and increased expression of PRL signaling inhibitors such as lif and stat3 (Campo Verde Arbocco et al., 2016).

PRL promotes mRNA synthesis of milk components such as β-casein (*csn2*) and α-lactalbumin (*lalba*) through binding to the long form of its receptor (PRLR). Mammary PRLR expression increases dramatically at the end of gestation, when lactation is established (Jahn et al., 1991; Nagano and Kelly, 1994; Wagner and Schmidt, 2011). PRL binding to PRLR induces receptor dimerization and activation of two signaling pathways, the JAK/STAT and PKB/AKT pathways, which allow transcription of specific milk proteins and inhibition of mammary involution (Jahn et al., 1997; Bole-Feysot et al., 1998; Schwertfeger et al., 2001; Creamer et al., 2010; Schmidt et al., 2014), the former pathway being the main signal transducer for milk component synthesis (Jahn et al., 1997; Anderson et al., 2007). The JAK/STAT pathway also induces expression of the SOCS/CIS protein family, that act as inhibitors of this pathway and participate in a negative feedback mechanism that limits PRL signaling.

The cellular context of lactating mammary ephitelial cells (MEC) activates signaling pathways that render the expression of a characteristic transcriptome known as "lactome" (lactation responsible genes proposed by Lemay et al., 2007) and becomes a biofactory of milk lipids, proteins and carbohydrates. This ability arises from massive suppression of other functions, which allow the MEC to redirect its functions primarily to the synthesis of milk components. In 2007, Lemay et al. (2007) described three main features of this extreme specialization. First, suppression of integrin signaling, which suggests that each cell is isolated from the rest to fulfill a single function. Second, suppression of the proteolytic machinery, suggests that the MEC acquires a large tolerance to proteins, to be able to store milk proteins. And third, the down regulation of the expression of proteins with signal peptide that will not be part of milk components. This occurs even up to the point of sacrificing the expression of the secretory machinery itself and enables the cell to synthesize and secrete large volumes of only a few proteins (Lemay et al., 2007). In this regard, any competing hormonal signals that tend to redirect lactating MECs to the expression of other metabolic pathways, may potentially hinder their ability to synthesize milk components in response to PRL.

Since THs are transcription factors closely related to tissue differentiation and metabolic function, HypoT can be considered a pathology that compromises cellular transcriptional capacity and the functionality of its signaling pathways. More specifically, THs have been shown to interfere with PRL signaling (Favre-Young et al., 2000), and through this mechanism they may interfere with mammary function. To continue investigating the mechanisms responsible for the lactational deficit observed in HypoT mothers, in the present work we investigated the impact of HypoT on the PRL signaling pathway expression and activity during early to mid-lactation in the rat, periods in which the differentiation of the mammary cell changes progressively in response to the increasing metabolic demands from the growing litter. Given the role of THs as transcription factors, we analyzed HypoT as a pathology that alters both transcriptional cell capacity and the functionality of its signaling pathways. For this purpose, we analyzed a part of the lactome directly involved in the synthesis of the major milk proteins in key stages of mammary cell differentiation.

2. Materials and methods

2.1. Experimental subjects

2.1.1. Animals

Adult female Wistar rats bred in our laboratory, 3–4 months old, weighing 190–210 g at the onset of treatment and with regular 4–5 day cycles, were used. The rats were kept in a light (lights on 06.00–20.00 h) and temperature (22–24 °C)–controlled room. Standard rat chow (Cargill, Cordoba, Argentina, composition provided in Supplementary Table 1) and tap water or 6-propyl-2-thiouracil (PTU) solution were available *ad libitum*. HypoT was induced by administration of PTU at a concentration of 0.1 g/L in drinking water. The treatment was started 8 days before the onset of pregnancy. Vaginal smears were taken daily and the rats were caged with a fertile male on the night of proestrus. The presence of spermatozoa in the vaginal smear the following morning was indicative of pregnancy, and this day was counted as day 0 of pregnancy. On day 1 of lactation, the number of pups in each litter was standardized to eight, thereafter, the pups remained with their mothers until the time of sacrifice. Mothers and litters were weighed weekly. Animal maintenance and handling was performed according to the NIH guide for the Care and Use of Laboratory Animals (NIH publication N8 86–23, revised 1985 and 1991), the UK requirements for ethics of animal experimentation (Animals Scientific Procedures Act 1986), and the FRAME guidelines of 1999. Groups of 8 PTU-treated (PTU) or control (Ctrl) rats were killed between 10.00 and 12.00 h on day 2, 7 and 14 of lactation (L2, L7 and L14) by decapitation. After decapitation, trunk blood was collected, and serum was separated by centrifugation and stored at −20 °C until used. Both inguinal mammary glands from the dams were removed, pooled, snap frozen in liquid nitrogen, and stored at −70 °C until they were analyzed. The procedures were approved by the Institutional Animal Care and Use Committee of the School of Medical Sciences, Universidad Nacional de Cuyo, Mendoza, Argentina (Protocol approval Nº 17/2012).

2.1.2. Cell culture

Mammary MCF7 cells were grown to 60% confluency and cultured with 10% charcoal stripped serum for 24 hs in Dulbecco's Modified Eagle Medium. After that, the cells were treated with T3 10^{-9} M (T3) and without T3 (No T3) for 24 hs. Cells were crosslinked with Formaldehyde 1% for 10 min and the reaction was stopped with glycine.

2.1.3. In-silico analysis

The *In-silico* analysis of rat *Prlr, Stat5a, Stat5b, Socs1, Socs2, Socs3,* and *Cis* genes promoter cores and human STAT5b gene to investigate the presence of thyroid hormone responsive elements (TRE) (in the rat and human genes), STAT responsive elements (GAS), progesterone responsive elements (PRE) and estrogen responsive elements (ERE) in the rat genes, was performed using the software "MatInspector" provided by Genomatrix™ (Genomatrix Software GmbH, Munich, Germany) and the NIH GenBank database (National Institutes of Health, Maryland - USA) according to Cartharius et al. (2005); Goldhar et al. (2011).

2.2. Hormone determinations

Serum TSH and PRL concentrations were measured by double antibody radioimmunoassay using materials provided by Dr. A.F. Parlow and the NHPP (National Hormone and Pituitary Program,

Harbor-UCLA Medical Center, Torrance, CA, USA) as previously described (Hapon et al., 2003).

2.3. RNA isolation and RT-Real time PCR analysis

Total RNA from mammary glands was prepared using the guanidinium isothiocyanate-acid phenol method as modified by Puissant and Houdebine (1990). Ten micrograms of total RNA were reverse transcribed at 37 °C using random hexamer primers and Moloney murine leukemia virus retrotranscriptase (Invitrogen-Life Technologies, Buenos Aires, Argentina) in a 20 μL reaction mixture. The RNA was first denatured at 70 °C for 5 min in the presence of 2.5 μg of random hexamer primers (Invitrogen). For the subsequent RT reaction the following mixture was added: RT buffer [50 mM Tris-HCl (pH8.4), 75 mM KCl, 3 mM MgCl2], 0.5 mM dNTPs, 5 mM DTT, 200 units M-MLV Reverse Transcriptase. The reaction was incubated at 37 °C for 50 min, next, the reaction was inactivated by heating at 70 °C during 15 min. The cDNA was stored at −20 °C.

The mRNA level of PRLR (*prlr*), *stat*5a, *stat*5b, suppressors of cytokine signaling 1, 2 and 3 (*socs* 1, 2, 3), cytokine-induced STAT inhibitor (*cis*), and milk proteins α-lactoalbumin (*lalba*) and β-casein (*csn2*) were estimated by RT Real Time PCR using rat-specific primers and reaction conditions described in Supplementary Table 2. The PCR reactions were performed using a Corbett Rotor Gene 6000 Real-Time Thermocycler (Corbett Research Pty Ltd Sydney, Australia) and Eva-Green™ (Biotium Hayward, CA) in a final volume of 20 μL. The reaction mixture consisted of 2 μL of 10× PCR Buffer, 1 μL of 50 mM MgCl2, 0.4 μL of 10 mM dNTP Mix (Invitrogen), 1 μL of 20× Eva Green, 0.25 μL of 5 U/μL Taq DNA Polymerase (Invitrogen) 0.1 μL of each 2.5 mM primer (forward and reverse primers) and 10 μL of diluted cDNA. The PCR reactions were initiated with 5 min incubation at 95 °C, followed by 40 cycles of 95 °C for 30 s, 30 s at the annealing temperatures shown in Supplementary Table 1 and 72 °C for 30 s. Melt curve analysis was used to check that a single specific amplified product was generated. Real time quantification was monitored by measuring the increase in fluorescence caused by the binding of EvaGreen™ dye to double-strand DNA at the end of each amplification cycle. Relative expression was determined using the Comparative Quantitation method of normalized samples in relation to the expression of a calibrator sample, according to the manufacturer's protocol (Pfaffl, 2001). Each PCR run included a no-template control and a sample without reverse transcriptase. All measurements were performed in duplicate. The reaction conditions and quantities of cDNA added were calibrated so that the assay response was linear with respect to the amount of input cDNA for each pair of primers. RNA samples were assayed for DNA contamination by performing the different PCR reactions without prior reverse transcription. To select the reference gene, we estimated the expression stability of four candidate reference genes, β-Actin, S16, GAPDH and HPRT1 using the freely available online software BestKeeper version 1 [http://gene-quantification.com/bestkeeper.html] as previously described (Campo Verde Arbocco et al., 2016; Campo Verde Arbocco et al., 2015). This approach allowed us to select S16 as the reference gene since it showed the lowest coefficient of variation between treatment groups compared to β-actin, GAPDH and HPRT1. Relative levels of mRNA were normalized to the S16 reference gene. The real time PCR products were analyzed on 2% agarose gels containing 0.5 μg/mL ethidium bromide and a unique band of the approximately correct molecular weight corresponded with a unique peak in the melt curve analysis.

2.4. Protein isolation and western blot analysis

Total proteins were extracted from mammary tissue by homogenization in 2 vol of homogenization buffer (50 mM Tris, pH 7.5, 250 mM sucrose, 10 mM benzamidine, 10 mM NaF, 5 mM sodium pyrophosphate, 20 mM glycerophosphate, 1 mM sodium orthovanadate, 1 mM PMSF, 10 mM p-nitrophenylphosphate, and aprotinin, leupeptin, and pepstatin at 2 mg/L) in an ice bath. The homogenate was centrifuged at 12.500 g for 30 min and the supernatant was separated and frozen in several aliquots at −80 °C until used. Proteins were quantified using the BCA method and boiled 3–5 min in loading buffer. 80 μg of protein were separated by SDS-PAGE and transferred to Nitrocellulose Hybond membranes as described previously (Valdez et al., 2007). After rinsing and blocking with BSA 0.5% the membranes were probed with anti-PRLR (ab2771, Abcam, Cambridge MA), anti-STAT5a/b (sc835), anti-P-STAT5a/b (sc11761), anti-SOCS3 (sc7009), and anti-CIS (sc15344) of Santa Cruz Biotechnology Inc, Dallas, TX, using horseradish peroxidase-conjugated secondary antisera (polyclonal goat anti-rabbit, rabbit anti-goat and goat anti-mouse inmunoglobulins, Dako Cytomation-CA) and chemiluminescence (Amersham ECL™, GE Healthcare, Buenos Aires, Argentina) to detect specific bands that were quantified by densitometry using FIJI Image processing package (Schindelin et al., 2012). We used densitometrical analysis of the Ponceau staining intensity of the transferred membranes as loading control.

2.5. Chromatin immunoprecipitation

For chromatin immunoprecipitation we followed the Chromatin Immunoprecipitation Protocol of Novus Biologicals (https://www.novusbio.com/support/support-by-application/chromatin-immunoprecipitation/protocol.html). Cells were washed twice with PBS and harvested with PBS plus protease inhibitors cocktail P8340 (Sigma-Aldrich, Inc.). To ensure that DNA fragments ranged from 200 to 600 bp, the nuclear solution was sonicated in an ice bath with four 15 s bursts at 60% amplitude, each separated by a 45 s period. DNA fragment size was verified by agarose gel electrophoresis. The resulting chromatin was incubated overnight with Anti-thyroid hormone receptor β1 protein (TRβ1) (sc738 Santa Cruz Biotechnology). A 50 μL fraction of this chromatin was stored as a control without antibody (-AC). Antibody-bound chromatin and control without antibody were precipitated with Protein A/G conjugated agarose beads (sc2003, Santa Cruz Biotechnology Inc, Dallas, TX), washed with gradient stringent buffers, and eluted with elution buffer as per the Novus protocol instructions. Beads were washed and eluted by shaking in buffer with proteinase K at 60 °C, overnight. The eluted solutions were incubated at 65 °C overnight to reverse cross-links. DNA was purified from the eluent using CTAB detergent and was analyzed by Real Time PCR with specific primers for the TRE located at 58,431, 58,419 and 58,371 base pairs of the origin of the human STAT5 gene according to *in silico* analysis (Cartharius et al., 2005). The primers were designed with Beacon Design software 7.92 (Premier Biosoft International, Palo Alto CA). The primers used were: P.58431: Sense: 5′-GGCATCAGCAAAGGAATA-3′ and Antisense: 5′-TCTTAAAGTGCTTGGATTACA-3′, P.58371: Sense: 5′-GCAGCTTCCTCTCATTCC-3′ and Antisense: 5′-TGACACAGGAGGCAGAAT-3′ and P.58419: Sense: 5′-TGCTACTTGTCTCATGTCCT-3′ and Antisense: 5′-AGAAGGGCAAACAGGGAT-3′. Primers for NCOR2 promoter was used as TRβ1 chromatin immunoprecipitation control as previously reported (Ayers et al., 2014).

2.6. Statistical analysis

Statistical analysis was performed using GraphPad Prism, and two way ANOVA followed by the Bonferroni post hoc test to compare any two individual means. When variances were not

homogeneous log transformation of the data was performed. Differences between means were considered significant at the $p < 0.05$ level. For TSH radioimmunoanalysis results, variances were not homogeneous after log transformation of the data, so we used the Kruskal Wallis test and Dunn's multiple comparison post-test.

3. Results

3.1. Effect of HypoT on circulating PRL and TSH in rats in early and midlactation

As we previously reported (Hapon et al., 2003), HypoT rats had higher TSH serum levels in the three days analyzed but PRL serum levels were not modified at any of these days (Table 1).

3.2. Effect of HypoT on mammary mRNA and protein levels of JAK/STAT signaling pathway members in early and midlactation

The normal MEC differentiation from early (L2) to mid-lactation (L14) produced similar fluctuations in the expression level of several of the mRNAs analyzed, as seen in the control group. Mammary mRNA contents of *prlr* and *stat5b* diminished from L2 to L7, but at L14 had rebounded to higher values than those of L2 (Fig. 1A). *stat5a* mRNA level showed a similar pattern as *prlr* but the difference between L7 and the other two days did not achieve statistical significance. These patterns were significantly altered by HypoT in a different way for each mRNA. HypoT decreased *prlr* mRNA level on L2 and the increase on L14 was significantly smaller (Fig. 1A). *Stat5a* mRNA content was similar on all three days, dampening the tendency observed in the controls at L7. *Stat5b* mRNA content, in contrast, was similar to controls on L2 and L7, but significantly smaller than controls on L14 (Fig. 1A).

The PRLR and STAT5a/b proteins expression patterns were different from the mRNAs. PRLR level decreased gradually through lactation with a significant difference between L2 and L14. HypoT significantly decreased the protein level in the three days (Fig. 1B). STAT5a/b protein levels in the control group tended to fall between L2 and L7 and fell significantly in L14. HypoT decreased STAT5a/b protein levels on L2 and L7 to values that were similar on the three days (Fig. 1B). The level of phospho-STAT5a/b (P-STAT5a/b) increased through lactation in the control group, with levels significantly higher in L14 compared to L2. In the HypoT group, P-STAT5a/b levels were similar to controls on L2, but decreased significantly to 25% and 12.5% of controls in L7 and L14 respectively (Fig. 1B).

3.3. Effect of HypoT on mammary mRNA and protein levels of inhibitors of JAK/STAT signaling pathway in early and midlactation

In control rats, the SOCS family members mRNA levels, showed an uniform pattern of variation, with an increase from L2 to L7 and a return on L14 to values similar to L2. This increase was strong for *socs1* and *socs3*, while the changes for *socs2* did not achieve statistical signification and for *cis* only the decrease from L7 to L14 was significant (Fig. 2A). These patterns were opposite to *prlr* and *stat5*a/b, suggesting that the negative feedback mechanism characteristic of this signaling pathway can be seen at the transcriptional level. HypoT had differential effects. *socs1* mRNA expression pattern was not affected by HypoT but the fluctuations in *socs2* were enhanced, resulting in higher mRNA levels on L7 compared with L2 and L14. However, the expression levels were not significantly different from the controls on any day analyzed. On the other hand, HypoT blocked the *socs3* mRNA increase in L7 resulting in a constant and low expression level throughout lactation while *cis* levels were not different from controls, the decrease from L7 to L14 was abolished (Fig. 2A).

SOCS3 and CIS proteins did not vary between L2 and L14 in controls. These expression profiles differ from their mRNA pattern and highlight post-transcriptional modifications in protein expression and/or altered protein degradation. HypoT significantly diminished the protein levels of both in L2 and of CIS also in L7.

3.4. Bioinformatic analysis of PRL signaling pathway members core promoter genes

To investigate whether the hypothyroid state can directly modulate gene expression of PRL signaling pathway members we performed an *in silico* analysis. Since HypoT alters the protein level of estrogen and progesterone receptors (ER and PR) (Campo Verde Arbocco et al., 2015), we searched for the presence of TRE, PRE, ERE and GAS sequences in the promoters of all the genes whose mRNA abundance was analyzed.

We found TRE sequences in rat *prlr*, *stat5a*, *stat5b*, *lalba*, *csn2* and *ccnd1* genes. PRE sequences are only present in prlr gene and ERE sequences in the *socs3* gene. GAS sequences are present in *prlr*, *stat5a*, *socs2*, *socs3*, *lalba*, *csn2* and *ccnd1* genes. Surprisingly we did not find GAS sequences in the *stat5b* rat gene promoter. All these results are summarized in Table 2.

We identified 6 TRE sequences in the human *stat5b* gene promoter and only 2 TRE sequences in the rat promoter (Cartharius et al., 2005). The two rat TRE sequences had a high potential to functionally bind TRβ (expressed by matrix similarity scores of 0.762 and 0.780 as described in Cartharius et al., 2005, Table 2).

3.5. Chromatin immunoprecipitation in mammary cells

The presence of TREs in the rat *Stat5b* promoter (Table 2) and its mRNA expression pattern opposite to that of the TRβ1 protein expression pattern throughout lactation (Campo Verde Arbocco et al., 2015), led us to hypothesize that it could be a transcriptional target of TH bound to TRβ1. To test this, we performed a chromatin immunoprecipitation assay with TRβ1 in the MCF7 mammary cell line, exploring TR-TRE binding of TREs selected from the *Stat5b* gene thought to contain potentially functional TREs (suggested by a matrix similarity greater than 0.794, Cartharius et al., 2005). These regions were at position 58,431 bp in the plus strand (ps), 58,371 in the minus strand (ms) and 58,419 (ms). Ncor-2 was used as a positive control, as it also binds TRβ1 in its promoter region (Ayers et al., 2014). As shown in Table 3, the TRE identified in the *Stat5b* promoter region 58,431 showed a higher binding to TRβ1 in absence of T3, while the TRE identified in the *stat5b* promoter region 58,371 showed higher binding in the presence of T3, suggesting that liganded TRβ1 preferentially binds to promoter region 58,371 while the unliganded receptor binds preferentially to region 58,431 (Table 3). In contrast to this, the 58,419 region was not

Table 1
Circulating TSH and PRL concentrations on days 2 (L2), 7 (L7) and 14 (L14 of lactation in control and hypothyroid HypoT, propylthiouracyl-treated) rats. Propylthiouracyl was administered in the drinking water at a concentration of 0.1 g/L. The results represent the mean ± SEM (n = 8 rats per group). *p < 0.05 compared with respective control group. There were significant effects of treatment and day of lactation for TSH and day of lactation for PRL (see Supplementary Table 3 for statistics).

Day	TSH (ng/ml)		PRL (ng/ml)	
	Control	HypoT	Control	HypoT
L2	0.51 ± 0.12	40.8 ± 3.9*	10.2 ± 4.6	17.8 ± 6.4
L7	0.39 ± 0.11	18.2 ± 4.0*	178.0 ± 78.2	184.9 ± 84.9
L14	0.23 ± 0.08	13.8 ± 1.9*	26.1 ± 6.6	37.3 ± 12.7

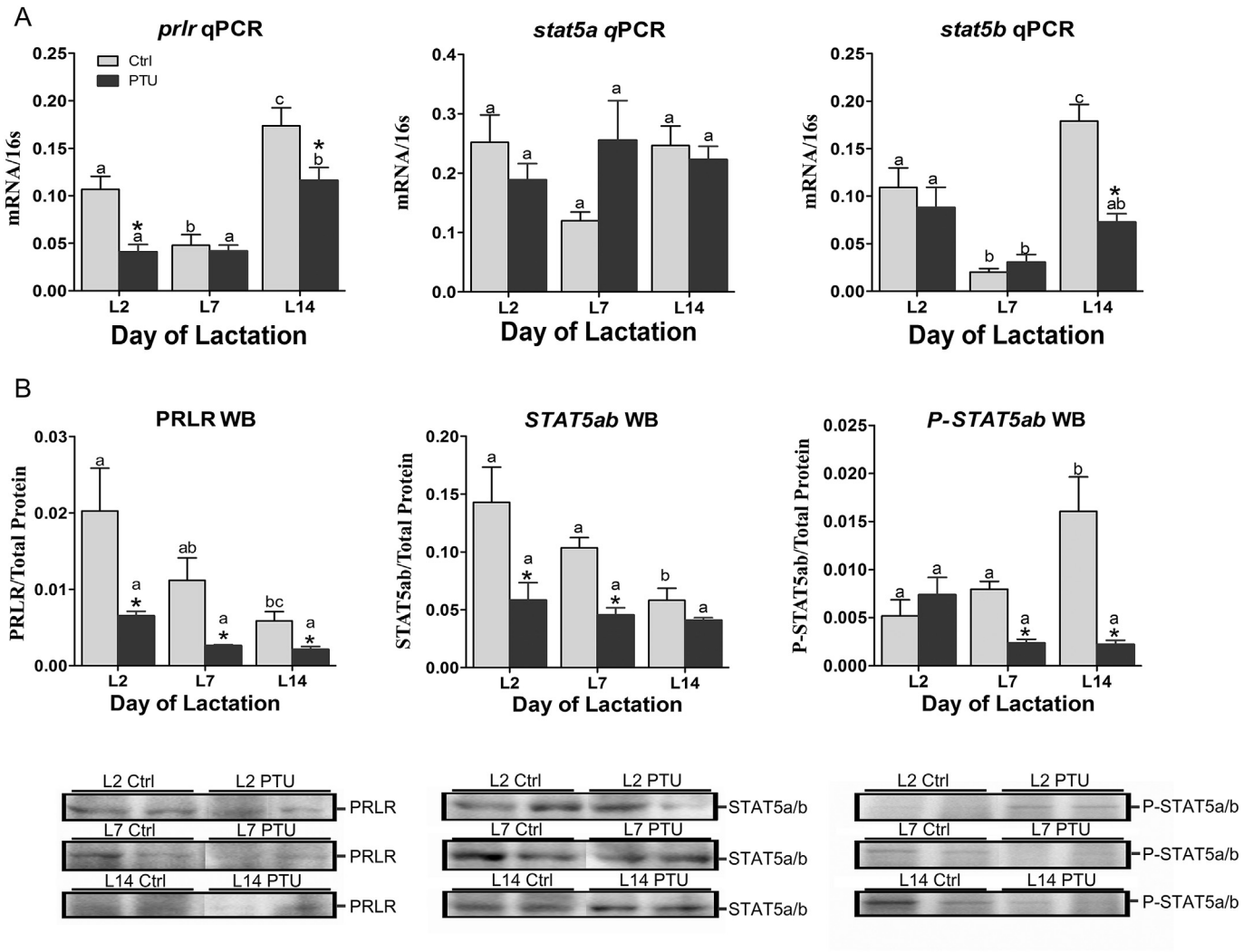

Fig. 1. Effect of PTU-induced hypothyroidism on mammary mRNA and protein levels of PRLR and JAK/STAT signaling pathway members during lactation. PRLR, STAT5a/b and P-STAT5 mRNA and protein levels were measured on days 2–14 of lactation. Controls (Ctrl, gray bars), Hypothyroid (PTU, black bars). mRNA levels, were measured by real time RT-PCR and expressed relative to S16 (Panel A). Protein levels were measured by western blot and expressed relative to total protein (Panel B); representative western blots for each day of lactation are shown below the corresponding graph. Values are means ± SEM for groups of 8 rats for PCR and 6 rats for WB. *$p < 0.05$ compared with the respective control group. Different superscript letters represent significant differences at $p < 0.05$ between different days of lactation within the same treatment groups. There were significant effects of treatment, day of lactation and interaction for *stat5b* mRNA and P-STAT5a/b protein, of treatment and day of lactation for PRLR mRNA and protein and STAT5a/b protein, *stat5a* mRNA showed no significant effects (see Supplementary Table 3 for statistics).

immunoprecipitated, suggesting that this region does not bind TRβ1 in this conditions. These results suggest that TRs, at least TRβ1, can directly modulate the expression of the *Stat5b* gene.

3.6. Effect of HypoT on mammary mRNA levels of some PRL pathway target genes in early and midlactation

We also studied the effect of HypoT on PRL action endpoints in lactation, such as the expression of the milk protein genes *csn2* and *lalba*, that codify for β-casein and α-lactalbumin respectively, and of *cnnd1*, that codifies for Cyclin D1, as a marker of cell proliferation. In the control group *lalba* and *csn2* mRNA levels increased from L7 to L14, but the increase was significant only for *lalba* (Fig. 3). This pattern was similar to the P-STAT5a/b pattern. In the HypoT group, the increase in *lalba* was blocked, with significantly lower levels in L14 compared with the control group. Again, this pattern was similar to P-STAT5a/b, *prlr* and *stat5b* between L7 and L14. HypoT had no significant effect on *csn2* expression (Fig. 3). The expression pattern of *cnnd1* showed a profile similar to *prlr* and *stat5b* with significantly lower values between L2 and L7, that increased in L14 to values not different from L2 or L7 in controls and HypoT deepened the drop in L7 making it significantly different from the other two days (Fig. 3), however, the values were not significantly different from the controls on the same days.

4. Discussion

It has been reported that at the end of the first week of lactation in rats, when the colostrum stage is over, litter growth increases the metabolic demand of the MG (Horst et al., 2005). In accordance with this, our results show an inflection point in L7 in the expression of most mRNAs studied, proving a significant change in the transcriptional activity of mammary cells. The mismatch between the patterns in mRNA and protein levels reveals that these are different biological entities and that HypoT alters each one differently. For example, the *ccnd1* mRNA expression profile confirms the differential expression of PRL target genes in each stage of mammary differentiation, and provides an example of plastic cellular

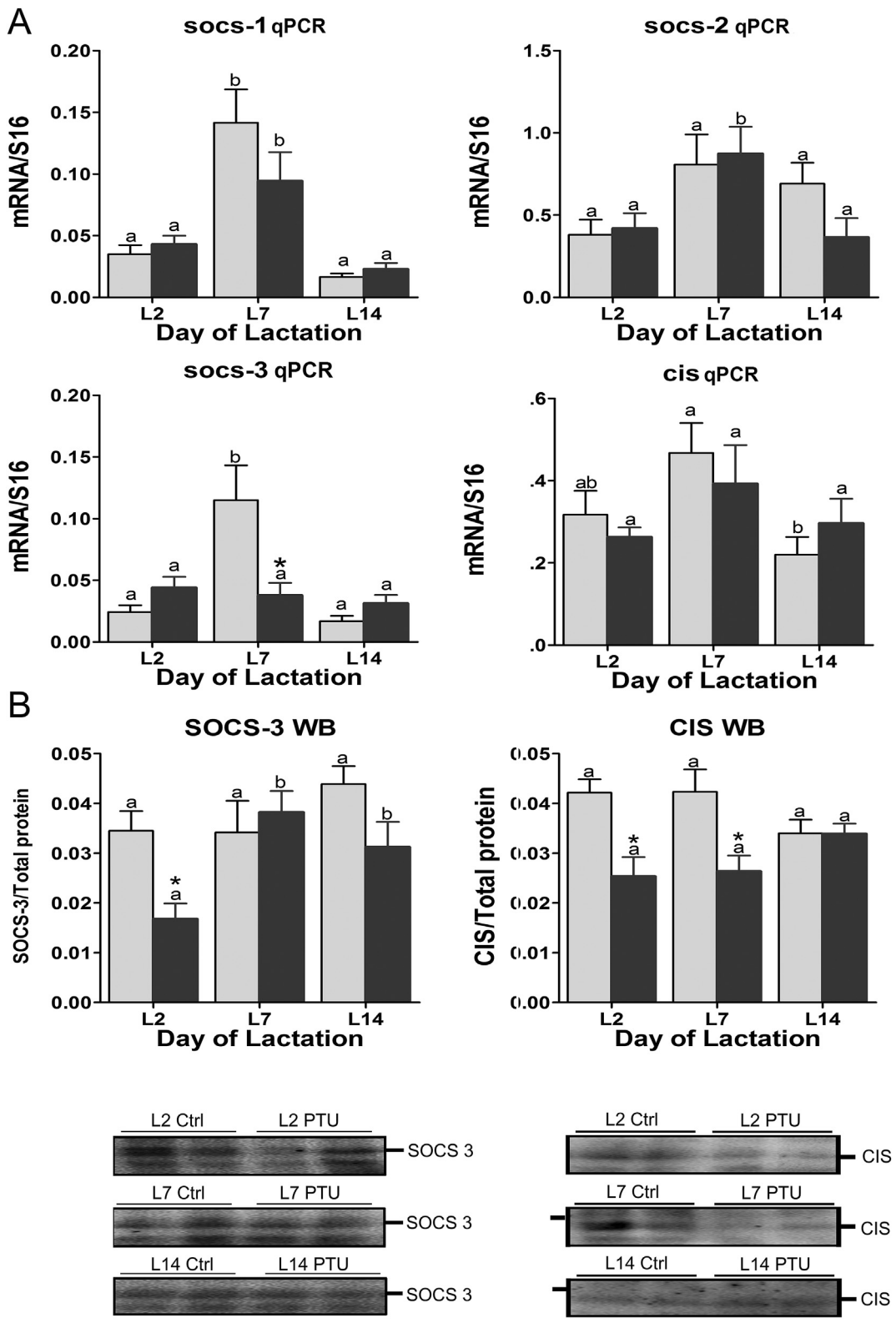

Fig. 2. Effect of PTU-induced hypothyroidism on mammary mRNA and protein levels of JAK/STAT signaling pathway inhibitors during lactation. The inhibitors of JAK/STAT signaling pathway members, SOCS1, SOCS2, SOCS3 and CIS, mRNA and protein levels were measured on days 2–14 of lactation. Controls (Ctrl, gray bars), Hypothyroid (PTU, black bars). mRNA levels were measured by real time RT-PCR and expressed relative to S16 (Panel A). Protein levels were measured by western blot and expressed relative to total protein (Panel B); representative western blots for each day of lactation are shown below the corresponding graph. Values are means ± SEM for groups of 8 rats for PCR and 6 rats for WB. *$p < 0.05$ compared with the respective control group. Different superscript letters represent significant differences at $p < 0.05$ between different days of lactation within the same treatment groups. There were significant effects of day of lactation for socs1, *socs2* and *cis* mRNAs, of day of lactation and interaction for *socs3* mRNA, of treatment and day of lactation for SOCS3 protein and of treatment for CIS protein (see Supplementary Table 3 for statistics).

Table 2
Bioinfomatic analysis of PRL signaling pathway members' core promoter genes. The search of binding regions for transcription factors on each promotor was performed with the Genomatrix MatInspector platform according to previously described procedures (Cartharius et al., 2005).

	TREs	PREs	EREs	GAS (1)	GAS (2)
Prlr	X	X		X	X
Stat5a	X				X
Stat5b	X				
Socs1					
Socs2					X
Socs3			X	X	X
Cis					
Lalba	X			X	X
Csn2	X			X	X
Ccnd1	X			X	
STAT5b (human)	X	X		X	X

TREs Thyroid hormone receptor response elements.
PREs Progesterone receptor response elements.
EREs Estrogen receptor response elements.
GAS(1) Stat5a/b response elements.
GAS(2) Stats other than Stat5ab response elements.

Table 3
Percentage of binding of TRβ to *Stat5b* promoter gene relative to input DNA. Chromatin immunoprecipitation (ChIP) experiments were performed by incubating lysates of MFC7 cells treated with T3 10^{-9} M (**T3**) or without (**No T3**) with anti-TRβ antibody for 24 h. The immunoprecipitated and isolated DNA was analyzed using real time PCR primers designed for three regions of *Stat5b* gene located 58431, 58371 and 58419 bases from gene origin. The region located at 58419 bases from gene origin did not amplify in PCR.

Location	No T3 (% Binding)	T3 (% Binding)	No Ab
58,431	2.450	0.006	0.001
58,371	0.389	9.754	0.005
Ncor-2	2.453	0.005	0.001

response dependent on both metabolic demand and environmental signals. In this regard, the drop of *ccdn1* mRNA level in L7 is a strong indicator of the overcoming of the colostrum stage since at L7 mammary alveolar expansion and maximum cell differentiation have been achieved, and mammary cells stop proliferating, focusing on the synthesis of milk components.

The present results demonstrate that HypoT has a negative effect on the PRLR/JAK/STAT5 signaling pathway, evidenced by a reduction of the mammary contents of *prlr* and *stat5b* mRNA, PRLR and STAT5a/b protein and particularly of P-STAT5. In turn, the reduction in PRL signaling may be responsible for the reductions in the expression of some PRL target genes such as *lalba* and *socs-3* and the previously observed decrease in milk lactose and lipids (Hapon et al., 2005).

Each stage of lactation has a transcriptome that results from the functional capacity of the MEC, the hormonal environment and metabolic requirements that are controlled by the intensity of the suckling stimulus provided by the litter (Lemay et al., 2007). These factors change dynamically as lactation proceeds. For example, in L2 or early lactation, the mRNA level of the PRL signaling main effectors is higher than at L7, maybe because the increased PRL and low progesterone (Hapon et al., 2003) promote mRNAs transcription and stabilization, thereby increasing the activity of the PRL pathway. In breast cancer cell lines, STAT5 and ERα can regulate transcription of *prlr* mRNA in a PRL and estrogen dependent process (Kavarthapu et al., 2014). Thus, the high STAT5a/b and P-STAT5a/b protein levels in L2 may promote mammary *prlr* mRNA transcription in early lactation. The changes in *prlr* mRNA level observed in the controls during lactation may be related to the changes in circulating progesterone (Hapon et al., 2003), progesterone receptor isoform B (PRB) and TRβ1 (Campo Verde Arbocco et al., 2015). Both receptor isoforms have been associated with inhibition of the transcriptional ability of STAT5 (Favre-Young et al., 2000; Hatsumi and Yamamuro, 2006) providing an inhibitory cellular context for *prlr* mRNA transcription, even with elevated PRL levels (Hapon et al., 2003). The PR binding sites present in the *prlr* gene promoter (Cartharius et al., 2005) could explain the inverse relationship between progesterone serum level and *prlr* mRNA level (Goldhar et al., 2011; Hovey et al., 2001; Kariagina et al., 2007, 2008; Morabito et al., 2008). On the other hand, transcription of *prlr* mRNA may be TH dependent, as suggested by the presence of TREs in its promoter and by the inhibitory effect of HypoT. We have shown that HypoT decreased TRα1 protein expression in L2 (Campo Verde Arbocco et al., 2015), an isoform closely related to differentiation of several tissues (Brent, 2000; Yen, 2001), and increased TRβ1 protein levels 20 fold (Campo Verde Arbocco et al., 2015), which could result in low *prlr* mRNA transcription through inhibition of STAT5 transcriptional activity (Favre-Young et al., 2000).

In the control group, the *stat5a*, *stat5b* and *prlr* mRNAs expression pattern throughout lactation may be related to mammary demand for this pathway. Although it has been described that STAT5a/b expression is under ER-E_2 regulation (Wang and Cheng, 2004), there is scarce research linking the transcription of these mRNAs to hormonal variations. Our *in silico* analysis shows that although the *stat5a* promoter has GAS sequences, the *stat5b* gene promoter analysis only showed TRβ response regions (Cartharius et al., 2005), that could act as a *cis* regulating element controlling its expression. The *stat5b* mRNA expression has a pattern opposite to the previously described TRβ1 protein pattern (Campo Verde Arbocco et al., 2015) and could be a result of inhibition by TRβ1 of *stat5b* transcription. Furthermore, the effect of HypoT on L14 that diminished *stat5b* mRNA levels and increased TRβ1 protein levels (Campo Verde Arbocco et al., 2015), strengthens the hypothesis that TRβ1, even in the presence of low circulating TH may inhibit the transcription of *stat5b* mRNA.

Although the *stat5a* gene promoter also contains TREs, the mRNA fluctuations along lactation and the impact of HypoT were not significant. Although both STAT5 isoforms have overlapping functions (Hennighausen and Robinson, 2008; Yamaji et al., 2009, 2013), STAT5a is the main transcription factor mediating lactogenic mammary gland function (Wagner and Schmidt, 2011; Hennighausen and Robinson, 2005, 2008) during lactation. According to this, it is not surprising that STAT5a expression is maintained, ensuring its correct expression and function even in pathological conditions such as HypoT.

The decrease in STAT5a/b and PRLR protein levels from L2 to L14, in the presence of elevated circulating PRL (Hapon et al., 2003) and unaltered or increased contents of their mRNAs, may be related to the proposal made by Lemay et al. (2007), that the milk synthesis machinery set up on late pregnancy is not replenished through lactation. This leads to its progressive degradation over time and consequently to a decrease in milk synthesis after peak lactation has been attained. Before L7 the proteolytic machinery expression in MEC is strongly inhibited but afterwards it is re-expressed, allowing the cell to degrade proteins and redirect its components towards milk synthesis (Lemay et al., 2007). Furthermore, the increased P-STAT5 may maintain the PRL stimulation of milk gene synthesis, even in the presence of reduced total STAT5 protein levels. On the other hand, the transcription pattern of the SOCS protein family mRNAs, in the control group could not be related to STAT5a/b protein level but, as for *stat5b*, was similar to the TRβ1 and PRB pattern and opposite to the PRA pattern (Campo Verde Arbocco et al., 2015). Although it has been shown that progesterone inhibits PRL lactogenic functions in late gestation, during lactation this inhibition is much weaker (Rosen et al., 1978; Murphy

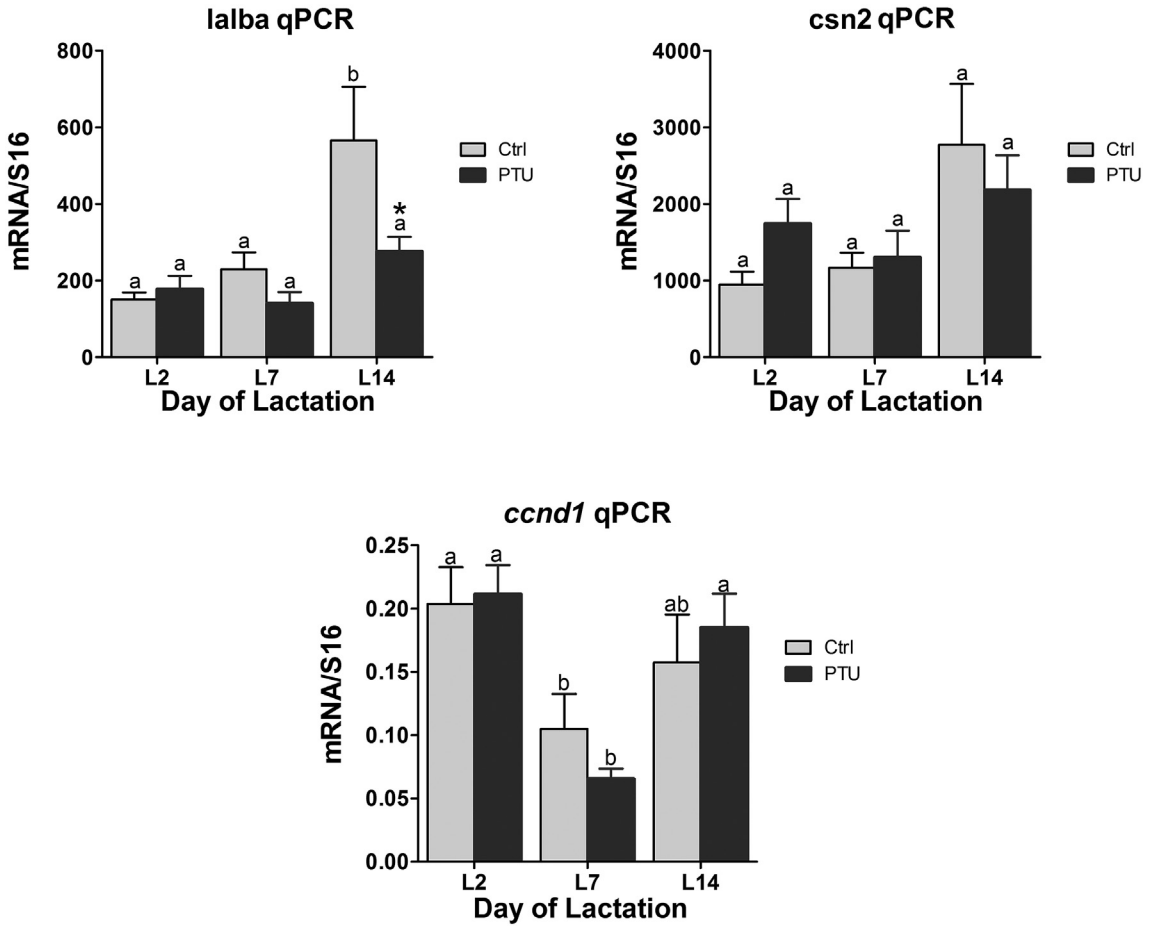

Fig. 3. Effect of PTU-induced hypothyroidism on mammary mRNA levels of the representative targets of JAK/STAT signaling pathway, β-casein (*csn2*), α-lactoalbumin (*lalba*) and Cyclin D1 (*ccnd1*) on days 2–14 of lactation. Controls (Ctrl, gray bars), Hypothyroid (PTU, black bars). mRNA levels were measured by real time RT-PCR and expressed relative to S16. Values are means ± SEM for groups of 8 rats. *$p < 0.05$ compared with the respective control group. Different superscript letters represent significant differences at $p < 0.05$ between different days of lactation within the same treatment groups. There were significant effects of day of lactation for *ccnd1* mRNAs, of day of lactation and treatment for *lalba* mRNA, of treatment and day of lactation for SOCS3 protein and *csn2* mRNA showed no significant effects for *csn2* mRNA (see Supplementary Table 3 for statistics).

et al., 1973). However, the increased mRNA expression level of the inhibitors of cytokine signaling was concomitant with maximum circulating progesterone (Hapon et al., 2003) and mammary PRB (Campo Verde Arbocco et al., 2015) suggesting that elevated progesterone action may promote their expression. HypoT abolished the *socs3* mRNA increase observed on L7, perhaps as a consequence of reduced PRL signaling and as a compensatory mechanism in order to preserve milk synthesis.

It is interesting to note that SOCS3 protein level is constant throughout lactation in the controls. The SOCS family proteins have differential capability for the inhibition of the different JAK/STAT signaling pathways, and SOCS3 particularly inhibits primarily STAT3 (Wagner and Schmidt, 2011; Robinson et al., 2007; Sutherland et al., 2007; Kreuzaler et al., 2011). In turn, STAT3 inhibits STAT5a/b activity and PRL signaling, so it may be expected that the levels of SOCS3 should be maintained through lactation. Conversely, the decreased SOCS3 protein induced by HypoT on L2 may increase STAT3 activity resulting in a less differentiated mammary phenotype (Hapon et al., 2007b). This may be another factor contributing to the lactation deficit of HypoT rats. The discrepancy between SOCS3 protein and mRNA levels is a strong indicator that their control mechanisms respond to different stimuli. In immune cells, SOCS3 translation is a rapid process that permits the immediate inactivation of the JAK/STAT pathway (Tamiya et al., 2011). Thus, the control of the protein level may lie mainly in its rate of degradation. However, since protein degradation is inhibited at this stage (Lemay et al., 2007) the mechanism by which HypoT decreased SOCS3 protein level may be exerted at pre-translational level. Similarly, CIS protein and mRNA levels were also constant throughout lactation, but HypoT decreased only the protein levels at L2 and L7. Since CIS over-expression inhibits the PRL signaling pathway (Fitzgerald et al., 2009), the fall in the protein levels in the HypoT group, could be a compensatory mechanism aiming to maintain activation of this signaling pathway. Alternatively, the inhibition of protein degradation observed in normal rats during the first half of lactation (Lemay et al., 2007) may be lifted prematurely in HypoT rats, since the levels of all the measured proteins were decreased.

Taking all this together, in HypoT the decrease in the inhibitor protein levels could be a compensatory cell response to the PRL signaling deficit caused by the fall of PRLR, STAT5 and P-STAT5, although it is important to note that in any case, this mechanism could not be maintained up to L14. According to the evolutionary importance of lactation it is probable that MEC are able to deploy compensatory responses to hormonal deficits in an attempt to maintain milk production. This compensatory response may explain why HypoT has no effect on *csn2* mRNA synthesis, which is the most abundant milk protein.

The *lalba* mRNA expression patterns in both groups correlated closely with the STAT5 activity and litter growth (Hapon et al.,

2003). The decreased mRNA level in L14 in the HypoT group may also be related with the increased TRβ1 on this day (Campo Verde Arbocco et al., 2015) since the *lalba* gene promoter has STAT5 and TRβ binding sites (Cartharius et al., 2005), and may be regulated by interaction between the PRL and TH signaling pathways (Favre-Young et al., 2000). α-Lactoalbumin is the lactose synthase cofactor controlling milk lactose synthesis. Lactose is not only the main milk carbohydrate, but also an osmotic regulator of milk volume. The fall on *lalba* caused by HypoT is most probably the cause of the low milk volume and lactose content of hypothyroid mothers described in Hapon et al., 2003.

Our results show that in L7 the lactome is subject to great changes. These are caused by the metabolic demand of the growing litter upon the MG and the hormonal environment of the first week of lactation, that lead to the establishment of mature lactation. Taking together, the present and our previous work (Campo Verde Arbocco et al., 2016; Campo Verde Arbocco et al., 2015) show that HypoT has a direct impact on some gene expression patterns of the lactating mammary gland, mainly on PRLR and its signaling pathway and on oxytocin receptor expression, which can be observed already on early lactation (L2) (Campo Verde Arbocco et al., 2015). PRL, oxytocin and their receptors are key components in the establishment of lactation (Rijnkels et al., 2010). It is at the early stages of lactation when the neuroendocrine control of milk synthesis is imprinting and the negative effects of HypoT on PRL signaling at these stages may be determinant for the course of the rest of lactation, resulting in reduced milk quality and quantity (Hapon et al., 2003) and premature mammary involution (Campo Verde Arbocco et al., 2016).

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.mce.2017.04.003.

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