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# STAT5 transcriptional activity is impaired by LIF in a mammary epithelial cell line

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**Abstract**

Gene regulation mediated by STAT factors has been implicated in cellular functions with relevance to a variety of processes. Particularly, STAT5 and STAT3 play a crucial role in mammary epithelium displaying reciprocal activation kinetics during pregnancy, lactation and involution. Here, we show that LIF treatment of mammary epithelial HC11 cells reduces the phosphorylation levels and transcriptional activity of p-STAT5 in correlation with STAT3 phosphorylation. We have also found that STAT5 activity is negatively modulated by this cytokine, both on a gene whose expression is induced, as well as on a promoter repressed by STAT5. Besides, our results show that lactogenic hormones increase LIF effect on gene induction without modifying STAT3 phosphorylation state. Our findings strongly suggest that there is crosstalk between STAT5 and STAT3 pathways that could modulate their ability to regulate gene expression.


*Keywords:* STAT5; STAT3; LIF; Prolactin; Mammary epithelium; Involution; Signal transduction; Gene expression; HC11

Most cellular processes are the result of complex and finely regulated cell responses to external stimuli that often result in a change in the pattern of gene expression. The signal transducers and activators of transcription (STAT) factors are critical for signaling from a myriad of cytokine receptors and growth factors [1]. Upon ligand binding, the corresponding receptor undergoes conformational changes in its cytoplasmic domain that induce activation of receptor-associated members of the Janus-activated kinase (JAK) family. The corresponding kinase mediates phosphorylation at specific receptor's tyrosine residues, which then serve as docking sites for STAT proteins. Once recruited to the receptor, STAT factors become also phosphorylated by JAKs, on a specific tyrosine residue [2]. This post-translational modification determines nuclear retention of dimeric STATs, that were shuttling between the cytoplasm and the nucleus [3,4]. Once in the nucleus, STAT dimmers bind to a consensus DNA-recognition motif in the promoter region of specific target genes and thereby regulate their transcription [5].

STAT factors are capable of transmitting both differentiative and proliferative signals, depending on the cellular context regulating many critical aspects of normal cell function, such as growth, survival, differentiation, and apoptosis [6]. In addition to its function in non-tumoral cell biology, deregulation of JAK–STAT pathway is frequently observed in many primary tumors and is critical for many oncogenetic processes [7].

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Seven proteins compose the STAT family in mammals [8]. STAT5 and STAT3 are critical in mouse mammary gland cyclical periods of cellular proliferation, differentiation, and regression [9]. Activation of the aforementioned STAT factors is tightly coordinated in this tissue, displaying a reciprocal pattern. STAT5 is strongly activated by prolactin (PRL) towards the end of pregnancy, persists in an activated state during lactation, and is rapidly inactivated after cessation of suckling [10]. On the contrary, STAT3 activation is hardly detectable during lactation, but is strongly induced by leukemia inhibitory factor (LIF) at the onset of involution [9,11]. While STAT5 is required for functional differentiation of the mammary epithelium during pregnancy and lactation, STAT3 is an essential mediator of apoptosis at involution [9–12]. It has been suggested that STAT5 could be regulated by STAT3 at the onset of involution, but the mechanisms involved in this regulation are not yet completely understood [12].

On the basis of the key role of STAT5 and STAT3 factors in such relevant processes, and its particular kinetics of activation/inactivation in mammary epithelium, the aim of this work was to study its interaction to elucidate if there is crosstalk between them. We worked on the widely used *in vitro* model, HC11 [13] mouse mammary epithelial cells, that respond to lactogenic hormones and LIF. We found that LIF treatment reduced the phosphorylation and transcriptional activity of STAT5, suggesting it would be negatively modulated by p-STAT3. We also presented evidence that LIF-dependent gene regulation is strengthened in a lactogenic hormonal context. Taken together, our findings strongly suggest that there is crosstalk between STAT5 and STAT3 that greatly affects their ability to regulate gene expression. Although we used a non-tumoral model, the results obtained in the present work, could also shed light on malignant transformation processes.

## Materials and methods

*Cell culture and hormone treatments*. HC11 cells (kindly provided by Dr. Nancy Hynes [13]) were cultured at 37 °C under humidified atmosphere with 5% $CO_2$. Cells were grown to confluence in RPMI 1640 medium (Sigma), supplemented with 10% FCS containing penicillin (100 IU/ml), streptomycin (100 μg/ml), glutamine (2 mM), 5 μg/ml insulin, and 10 ng/ml EGF, and were maintained for 3 days in EGF depletion medium, rendering them receptive to stimuli of lactogenic hormones [13]. The competent cells were then washed and incubated for 18 h in serum-free medium, then treated for the indicated times with serum-free medium supplemented with the lactogenic hormones (indicated as PDI): 5 μg/ml of ovine prolactin (Sigma), $10^{-8}$ M of dexamethasone (Sigma), 4 μg/ml insulin (Sigma), with 5 ng/ml LIF (Sigma) or both, for the time indicated in each figure.

*Transient transfection and luciferase assay*. For transient transfection, $5 \times 10^5$ HC11 cells plated in 60-mm plates were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Cells were transfected with 3 μg of the reporter vector P1-*bcl-X*-LUC [14] and cotransfected with constitutively active STAT5a, STAT5b or both expression vectors, as indicated; or with the corresponding empty vector (kindly provided by Dr. Toshio Kitamura [15]). A β-galactosidase expression vector, pCMV-LacZ, was used as a transfection control. Forty-eight hours after transfection, cells were harvested with Cell Culture Lysis Reagent E153A (Promega). Luciferase activity was measured with a luciferase kit according to the manufacturer's protocol (*Luciferase Assay System* E1501, Promega). β-Galactosidase activity was measured as previously described [16].

*RNA preparation and RT-PCR*. Total RNA was isolated from HC11 cells as described previously [17] and was quantified spectrophotometrically. For reverse-transcription, 8 μg of total RNA, superscript-reverse transcriptase (Life Technologies, Inc.), and 25 ng/μl oligo(dT) (Life Technologies, Inc.) were used. For PCR amplification the following oligonucleotides were used: *β-casein* (forward): 5′-TCCCACAAAACATC CAGCC-3′, (reverse): 5′-ACGGAATGTTGTGGAGTGG-3′. *P1-Bcl-X* (forward): 5′-CCTGAAGCTCTCTCTCTCTCTTCA-3′, (reverse): 5′-CC AGCTCGGTTGCTCTGAGACAT-3′. *c-fos* (forward): 5′-TCCCTGGA TTTGACTGGAGGTCTG-3′, (reverse): 5′-ACAGCTTGGGAAG GAGTCAGC-3′. *clebp-δ* (forward): 5′-ACCCGCGGCCTTCTACGA-3′, (reverse): 5′-CGCCCCTTTTCTCGGACTGT-3′. The reactions yielded 450, 171, 400, and 486 bp length cDNA fragments, respectively. Annealing conditions for PCR reactions were 54 °C for 45 s, 57 °C for 40 s, 68.7 °C for 45 s, and 56 °C for 60 s, respectively. All PCRs were normalized against *actin* expression using the following primers: (forward): 5′-GTGGGCCGCTCTAGGCACCA-3′ and (reverse): 5′-CG GTTGGCCTTAGGGTTCAGGGGGG-3′. The reaction yielded a 250-bp-length cDNA fragment and the annealing conditions were 61 °C for 30 s. The PCR products were visualized by electrophoresis on 1.5 or 2% agarose gels containing ethidium bromide.

*Protein preparation and Western blot analysis*. Preparation of whole cell lysates and immunoblot analyses were described previously [16]. Briefly, protein was extracted from HC11 cells with RIPA *Buffer* supplemented with protease and phosphatase inhibitors (1 mM EGTA; 1 mM PMSF; 2 μg/ml pepstatin A; 10 μg/ml Leupeptin; 1 mM $Na_3VO_4$ and 1 mM NaF). After sonication, the lysates were centrifuged at 4 °C for 30 min at 12,000 rpm. Protein concentration in the supernatant was determined by the Bradford assay [18]. For detection of p-STAT5, total STAT5 was immunoprecipitated from 600 μg of whole cell lysates with specific antibodies. For detection of p-STAT3, 40–80 μg of whole cell lysates were used. The immune complexes or protein extracts were subjected to 9% SDS–PAGE, transferred to nitrocellulose membranes, and analyzed by Western blot. Protein bands were visualized by incubating with a peroxidase-conjugated secondary antibody antirabbit IgG or antimouse IgG (Bio-Rad) followed by enhanced chemiluminescence with detection system, ECL (Amersham Pharmacia). The proper loading was evaluated by staining the membranes with Ponceau-S. The antibodies used in this study were against (pTyr 705)-STAT3, STAT3, STAT5 (catalog Nos. sc-8059, sc-482, and sc-836, respectively, from Santa Cruz Biotechnology, Inc.), and anti-pTyr (catalog No. 05-321, Upstate Biotechnology, Inc.).

## Results and discussion

### *LIF treatment induces STAT5 dephosphorylation in correlation with STAT3 phosphorylation*

Based on the evidence of the particular kinetic of activation of STAT5 and STAT3 in mammary epithelium [9,12], we wondered if there is a crosstalk between these factors. We first analyzed the levels of p-STAT5 in the presence of the cytokine LIF, which is produced at the onset of post-lactational involution and activates STAT3 [9,11,12]. We incubated competent HC11 cells during 15 and 45 min with LIF, and analyzed the phosphorylation state of STAT5 by Western blot. LIF treatment drastically reduced the levels of p-STAT5 without altering the amount of total STAT5 (Fig. 1A). Remarkably, in the same protein extracts, STAT3 became phosphorylated upon LIF

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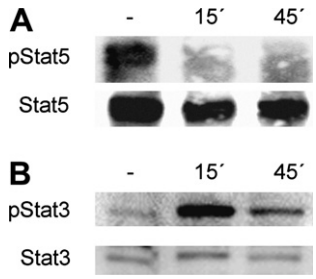


Fig. 1. LIF treatment induces STAT5 dephosphorylation in correlation with STAT3 phosphorylation. Competent HC11 cells were treated with LIF for 15 or 45 min or left untreated (−). (A) Equal amounts of proteins from each group of cell lysates were immunoprecipitated using antibodies against STAT5 and subjected to immunoblot analysis. Membranes were probed with a monoclonal antibody against pTyr (upper) and later reprobed with antibodies against STAT5 (lower). (B) Equal amounts of proteins from each group of cell lysates were analyzed by Western blot. Membranes were probed with antibodies against (pTyr 705)-STAT3 (upper) and later reprobed with antibodies against STAT3 (lower). Results are representative of two independent experiments.

addition in an opposite manner, showing increased phosphorylation levels after 15 min treatment (Fig. 1B). This reciprocal phosphorylation pattern strongly indicates a high correlation between LIF-induced STAT3 phosphorylation and STAT5 dephosphorylation. This result is in accordance with the p-STAT5 decline existing at the onset of involution of the mammary gland, when LIF levels increase [19,20]. We have also observed that after 45 min of treatment, STAT5 remained appreciably dephosphorylated, even when the levels of p-STAT3 diminished. This fact suggests that, if p-STAT3 is required for STAT5 dephosphorylation, minimal levels of p-STAT3 are sufficient to maintain STAT5 in an unphosphorylated state.

### *LIF treatment prevents β-casein induction by lactogenic hormones*

STAT activity is regulated at multiple levels by different factors that modulate its transcriptional regulation capability, either affecting its phosphorylation state or without changing it [21]. We wondered whether the change in STAT5 phosphorylation as a consequence of LIF treatment affected the transcription factor activity. We analyzed, by RT-PCR assays, the mRNA levels of *β-casein,* a widely used STAT5 target gene [11,22]. Notably, incubation of HC11 competent cells with LIF not only diminished the basal levels of *β-casein* mRNA, but also abolished *β-casein* induction by the lactogenic hormones prolactin, dexamethasone, and insulin (PDI) (Fig. 2A). These results were obtained with hormone treatments of 6 h and confirmed by Western blot analysis of *β-casein* protein levels after 72 h of treatment (data not shown). Similar results were obtained with 8 h of hormone treatment (data not shown). These evidences demonstrated that besides causing STAT5 dephosphorylation, LIF treatment abolished STAT5 action on gene transcription. Moreover, this effect persisted in the presence of lactogenic hormones.

### *STAT5a and/or STAT5b repress bcl-X P1 promoter directed expression*

To extrapolate these findings to other genes, we looked for another STAT5 target gene. *Bcl-X* gene bears at least five promoters (P1–P5) that are activated in a tissue specific manner [14]. There are two putative STAT5 binding sites in P1 region, similar to those found in *β-casein* promoter [23].

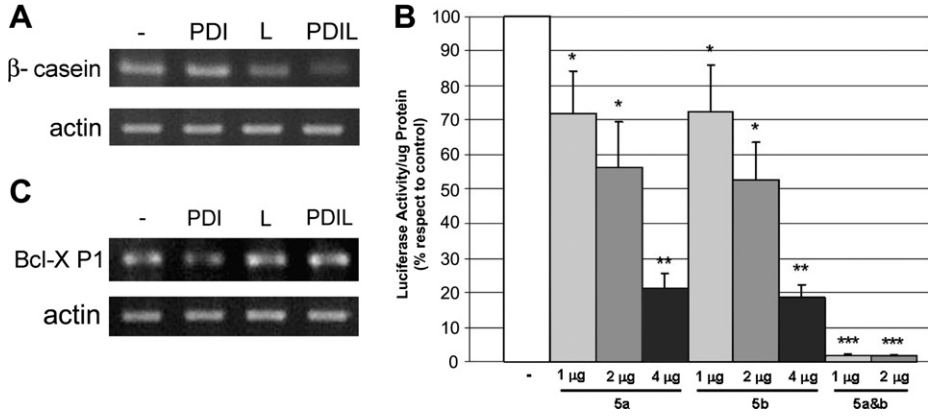


Fig. 2. LIF treatment prevents β-casein induction and *bcl-X* P1 repression by lactogenic hormones. (A) Competent HC11 cells were subjected to 6 h of lactogenic hormone (PDI), LIF or both (PDIL) treatments or left untreated (−) for the same period of time. Equal amounts of RNA isolated from each group of cells were subjected to RT-PCR using specific primers to analyze β-casein mRNA (upper) or actin mRNA (lower). Each experiment was performed twice independently with comparable results. (B) HC11 cells were transiently transfected with 3 μg of the reporter vector P1-*bcl-X*-LUC and cotransfected with 1, 2 or 4 μg of constitutively active STAT5a (5a) or STAT5b (5b) or both (5a and b) expression vectors, as indicated; or cotransfected with the corresponding empty vector (−). Three independent experiments were performed by duplicate. Luciferase activity measures were normalized with the registered β-galactosidase activity and total protein content. Each bar represents the mean percentage of the control value ± SEM. Student's *t* test was used to reveal significant differences between the groups. Bars with a single asterisk are significantly different to the control; bars with two asterisks are significantly different to the ones with a single asterisk and bars with three asterisks are significantly different to the bars with two asterisks ($p < 0.05$). (C) Competent HC11 cells were subjected to 6 h of lactogenic hormone (PDI), LIF or both (PDIL) treatments or left untreated (−) for the same period of time. Equal amounts of RNA isolated from each group of cells were subjected to RT-PCR using specific primers to analyze P1-*bcl-X* mRNA (upper) or actin mRNA (lower). Each experiment was performed twice independently with comparable results.

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We evaluated the possibility that STAT5 could regulate P1-driven gene expression in mammary epithelium cell culture; thus HC11 cells were transiently cotransfected with constitutive active STAT5a and/or STAT5b mutants [15] with a P1 reporter construction, P1-*bcl-X*-luc [14]. Overexpression of both STAT5a and STAT5b repressed P1-directed luciferase gene expression in a dose-dependent manner (Fig. 2B). These results evidence the functionality of P1-*bcl-X* STAT5 binding sites. So, we could exploit this promoter as an additional reporter of STAT5 activity, in which it has the opposite effect than that elicited on the *β-casein* gene.

Interestingly, cotransfection with both STAT5a and b, achieved a greater level of gene repression, suggesting that the heterodimer STAT5a/STAT5b could be a more effective regulator of this promoter than the corresponding homodimers. This effect agrees with the fact that, in spite of the low levels of STAT5b in mammary tissue, many genes are regulated by STAT5a/STAT5b heterodimer [20].

### *Lactogenic hormones inhibit P1-bcl-X directed expression and LIF treatment prevents this repression*

Having established that STAT5 is able to repress P1 transcriptional activity, we analyzed the effect of lactogenic hormone treatment on P1-driven expression. In accordance with our previous results, we found that PDI greatly repressed P1-*bcl-X*-derived transcript (Fig. 2C), suggesting that endogenous STAT5 regulates negatively this promoter activity. Supporting our hypothesis, LIF treatment prevented repression exerted by PDI, since simultaneous incubation of the cells with PDI and LIF exhibited P1-*bcl-X* mRNA levels similar to those of the untreated control and considerably higher than those obtained with PDI treatment. Results shown in Fig. 2C were obtained with 6 h treatments. Similar results were obtained with 8 h of hormone treatment (data not shown).

In summary, we have demonstrated the existence of a negative outcome of LIF on STAT5 phosphorylation and also a repressive effect on PDI action, both on a gene whose expression is induced, as well as on a promoter repressed by STAT5.

The high correlation shown by our results strongly supports the hypothesis that LIF treatment, presumably by activation of STAT3, counteracts PDI-gene regulation in mammary epithelium, most probably through down-regulation of STAT5 activity. Thus, it is tempting to speculate that negative modulation of STAT5 activity at the onset of post-lactational involution could be an outcome of STAT3 activation by LIF, a critical event for the mammary epithelium involution process.

We have shown for the first time that LIF treatment negatively modulates STAT5 activity and phosphorylation *in vitro*. In recent reports it has been proposed that STAT5 activity could be downregulated by STAT3 [12] and that STAT5 activity could be suppressed by the transcriptional effects of STAT3 at the onset of involution as part of the apoptotic program [11]. LIF-induced STAT5 activity down-regulation can be explained, at least in part, by the documented STAT3 transcriptional effect on SOCS3 gene [11]. Conversely, in a recent work, it was proposed that the cytoplasmic tyrosine phosphatase Shp2, may have dual functions in regulating STAT5 activity in the mammary gland, first acting as a positive regulator for the activation of STAT5 by prolactin and then as a negative effector in dephosphorylation/down-regulation of the activated STAT5 pathway [24]. Even so, future investigation will be required to address the identity of the process involved.

### *Lactogenic hormones favour LIF induction of c-fos and C/EBP-δ without modifying STAT3 phosphorylation state*

Having determined that LIF treatment could prevent PDI regulation of gene expression, we further investigated if reciprocally, lactogenic hormones have any effect on STAT3 activity. To address this question, we analyzed STAT3 activity on gene regulation by measuring mRNA levels through RT-PCR of two STAT3 target genes, *c-fos* and *C/EBP-δ* [11]. As expected, LIF induced *c-fos* gene expression but surprisingly, cell treatment with both LIF and lactogenic hormones, produced an evident further increase in *c-fos mRNA* levels (Fig. 3A). Analysis of *C/EBP-δ* mRNA levels confirmed this finding (Fig. 3B). This suggests that STAT3-mediated gene induction is strengthened in a lactogenic context. Nevertheless, we cannot rule out that lactogenic hormones were directly regulating *c-fos* and *C/EBP-δ* gene expression. It has been reported

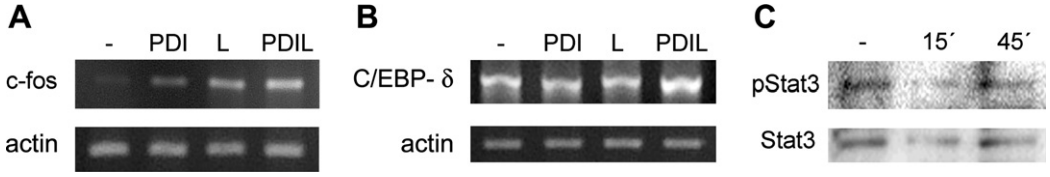


Fig. 3. Lactogenic hormone-treatment of HC11 cells enhances LIF induction of c-fos and C/EBP-δ, without modifying STAT3 phosphorylation state. Competent HC11 cells were subjected to 6 h of lactogenic hormone (PDI), LIF or both (PDIL) treatments or left untreated (−) for the same period of time. Equal amounts of RNA isolated from each group of cells were subjected to RT-PCR using specific primers to analyze *c-fos* mRNA (A, upper), *C/EBP-δ* mRNA (B, upper), or actin mRNA (A and B, lower). Each experiment was performed twice independently with comparable results. (C) Competent HC11 cells were treated with LIF for 15 or 45 min or left untreated (−). Equal amounts of proteins from each group of cell lysates were analyzed by Western blot. Membranes were probed with antibodies against (pTyr 705)-STAT3 (upper) and later reprobed with antibodies against STAT3 (lower). Results are representative of two independent experiments.

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that *c-fos* gene expression is modulated by insulin and dexamethasone [25] and that some members of *C/EBP* family are regulated by prolactin, dexamethasone and insulin in a variety of cellular types [26]. However, there is no evidence that these regulation processes could take place in mammary epithelium. Moreover, despite *C/EBP*α and β activities are regulated by lactogenic hormones in HC11 cells, it was demonstrated that their protein levels were not affected [27].

Therefore, according to these data, stimulatory action of LIF on STAT3 activity is augmented in the presence of lactogenic hormones, on at least two STAT3 target genes. The results shown for both genes were obtained with hormone treatments of 6 h. Similar results were obtained with 8 h of hormone treatment (data not shown).

To assess the possibility that the increase in STAT3 target genes expression in the context of both LIF and lactogenic hormones is a consequence of an increase in the phosphorylation of STAT3 promoted by PDI treatment, we analyzed through Western blot assays, the levels of p-STAT3 in the presence of lactogenic hormones. The phosphorylation state of STAT3 did not change either after 15 min or after 45 min of PDI treatment (Fig. 3C). This observation agrees with the evidence that p-STAT3 is maintained at a low level, when there are high concentrations of circulating lactogenic hormones, during mammary epithelium proliferation and differentiation [12,22]. As a whole, the above results indicate that despite STAT3 phosphorylation is not modulated by lactogenic hormones, they increase LIF effect on gene induction. Therefore, we propose that there could be other PDI-dependent factors that might contribute with STAT3 on increasing gene expression. This proposal would be consistent with the fact that in early involution plasma levels of lactogenic hormones remains detectable [28].

Taken together, our results show that on one hand, PDI-dependent gene regulation is impaired by LIF treatment and, on the other hand, lactogenic hormonal context enhances LIF action on gene expression modulation. Hence, we propose that there is crosstalk between STAT5 and STAT3 pathways that greatly affects their ability to regulate gene expression.

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