LIF, a Novel STAT5-Regulated Gene, Is Aberrantly Expressed in Myeloproliferative Neoplasms

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Abstract

A search for genes potentially regulated by STAT5 identified leukemia inhibitory factor (*LIF*) as a good candidate. Using various experimental approaches, we have validated *LIF* as a direct transcriptional target of STAT5 in myeloid cell lines: STAT5 binds to *LIF* promoter, and *LIF* expression is increased after activation of the JAK2/STAT5 pathway. We also found that *LIF* expression is significantly increased in patients with chronic myeloproliferative neoplasms with and without activating mutations of the pathway, indicating that *LIF* might play an important role in STAT5-mediated oncogenesis.

Keywords

STAT5, LIF, myeloproliferative neoplasms

Introduction

The Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathway plays a crucial role in myeloid differentiation. Binding of cytokines and growth factors to membrane receptors activates a JAK nonreceptor tyrosine kinase that phosphorylates a specific STAT transcription factor. Once phosphorylated, STATs migrate to the nucleus and bind to specific consensus sequences in the promoter of target genes involved in various cellular processes such as proliferation and apoptosis. This pathway is frequently altered in BCR/ABL1–negative chronic myeloproliferative neoplasms (CMPNs) by activating mutations in *JAK2*.[1] However, constitutive activation of the pathway by the same mutation (V617F) leads to the development of MPNs with distinct phenotypic features.[2] Although some variables (such as V617F allele burden, other mutations in *JAK2*, or mutations in other genes such as *TET2*) have been proposed to explain this fact, none of them fully accounts for the observed phenotypic differences in this group of diseases. This suggests that downstream effectors of JAK-STAT signaling could play a significant role in STAT5-mediated oncogenesis and might account for this unexplained phenotypic variability.

In order to identify such downstream effectors, we performed a genome-wide bioinformatic search for the presence of STAT5-binding sites in human promoters. This identified leukemia inhibitory factor (*LIF*) as a potential candidate gene to be transcriptionally regulated by phosphorylated STAT5. Because *LIF* codes for a multifunctional cytokine that plays an important role in hematopoietic differentiation[3] and in the maintenance of the pluripotent state of stem cells,[4] expression changes of this gene might be important in the pathogenesis of CMPNs. Thus, we set out to confirm the status of *LIF* as a direct transcriptional target of STAT5.

Results and Discussion

In order to validate the status of *LIF* as a *bona fide* STAT5-regulated gene, we used qRT-PCR to measure *LIF* expression changes in myeloid cell lines after activation of the JAK2/STAT5 pathway with interleukin-3 (IL-3). M07e cells were starved of growth factors for 16 hours and treated either with IL-3 alone or with a combination of IL-3 and a specific STAT5 inhibitor (cat. no. 573108, Calbiochem, San Diego, CA). As shown in Figure 1A, *LIF* expression was upregulated by IL-3 more than 20-fold, and this effect was

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reversed by treatment with STAT5 inhibitor. Similar changes were observed for STAT5 phosphorylation and for expression of other STAT5-regulated genes (Suppl. Results).

To confirm whether STAT5 binds to the promoter of *LIF*, we compared the amount of DNA-bound STAT5 before and after IL-3–mediated induction of the JAK2/STAT5 pathway in M07e cells. To this end, we performed chromatin immunoprecipitation (ChIP) with a specific STAT5 antibody and measured the amount of STAT5-bound DNA by quantitative PCR. We obtained an 11-fold increase in STAT5 binding to one site in the promoter of *LIF* (a TTCCCAGAA motif located 740 nucleotides upstream from the transcription start site) in IL-3–treated cells compared to untreated cells (Fig. 1B). Taken together, these results indicate that *LIF* behaves as a direct transcriptional target of STAT5 in this experimental model.

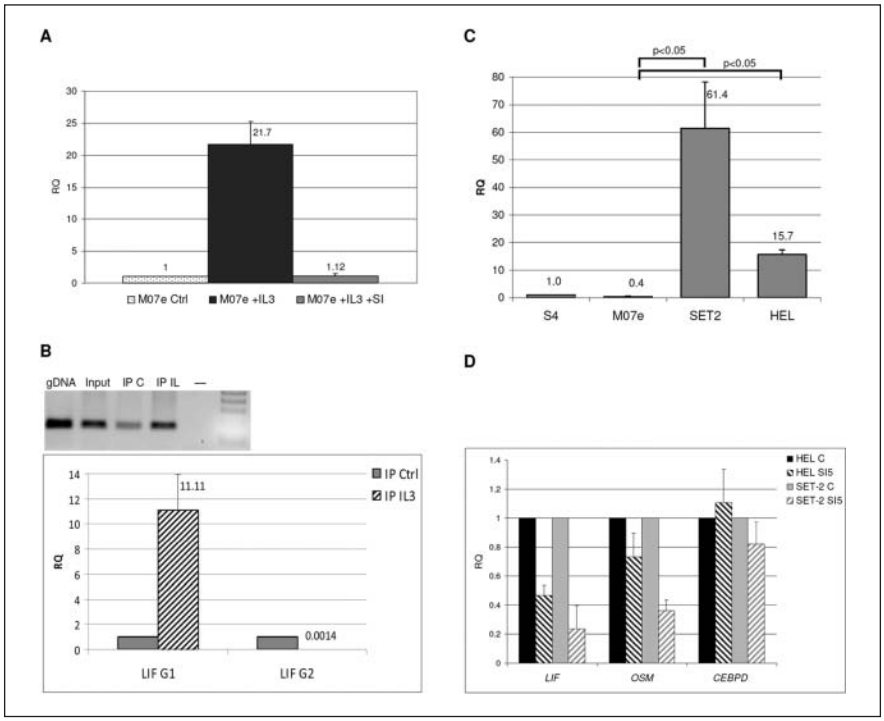

Figure 1. *LIF* is a direct transcriptional target of STAT5. (**A**) *LIF* expression is increased in M07e cells after activation of the JAK2-STAT5 pathway with IL-3. This was prevented by the addition of a specific STAT5 inhibitor (SI). Untreated control cells (RQ = 1) were used to normalize expression values. (**B**) Chromatin immunoprecipitation shows increased binding of STAT5 to a specific DNA motif in *LIF* promoter in IL-3–treated M07e cells (IP IL-3) compared to nonstimulated cells (IP Ctrl). Quantitative qPCR assays (bottom) confirm increased binding to a motif (G1) located 740 bp upstream of the transcription start site (TSS) but not to another putative binding motif (G2) located 154 bp upstream of the TSS. Input DNA (RQ = 1) was used to normalize values. An agarose gel of the PCR products (only for motif G1) is shown at the top. (**C**) *LIF* expression is increased in cell lines carrying the V617F activating mutation of *JAK2* (HEL and SET-2) compared to cell line M07 and to peripheral blood from a healthy control ($P < 0.05$). (**D**) Treatment of *JAK2*-mutated cell lines HEL and SET-2 with a specific STAT5 inhibitor reduces the basal expression levels of *LIF* and *OSM* without affecting the expression of the STAT3-regulated gene *CEBPD*. For each gene, results show fold inhibition compared to untreated cells (RQ = 1).

We next wanted to confirm whether *LIF* is regulated by activation of the JAK2/STAT5 pathway in other cell line models. To this end, we measured *LIF* expression in cell lines harboring the activating V617F mutation in *JAK2*. Compared to M07e, which does not carry this mutation, *LIF* expression was increased 16-fold in HEL cells and 61-fold in SET-2 cells, as shown in Figure 1C. Furthermore, we studied the effect of suppressing the activity of the pathway in these 2 cell lines using the specific inhibitor. Incubation of HEL and SET-2 cells with STAT5 inhibitor decreased *LIF* mRNA levels (2.1-fold decrease in HEL and 4.2-fold decrease in SET-2), as shown in Figure 1D. We also measured expression of *CEBPD*, a gene known to be regulated by STAT3 that lacks STAT5-binding sites in its promoter. As a further confirmation of the specificity of this inhibitor for STAT5, *CEBPD* expression was not affected by treatment of HEL and SET-2 cells (Fig. 1D). These data confirm that *LIF* expression is directly regulated by the induction of JAK2/STAT5 signaling in myeloid cell lines.

Our results are in agreement with several datasets available from the literature, in which gene expression has been monitored during myeloid differentiation. In one study,[5] cord blood CD34+ hematopoietic progenitor cells were differentiated through erythroid, megakaryocytic, granulocytic, and monocytic pathways at different time points. Their data support the notion that *LIF* is upregulated in erythroid, megakaryocytic, and granulocytic lineages, with particularly marked expression changes in the latter. Another recent study[6] used an estrogen receptor–STAT5 fusion to induce STAT5 and monitor gene expression in human hematopoietic stem cells and in myeloid progenitor cells. In this dataset, *LIF* expression was significantly upregulated in GMP and HSC after STAT5 induction.

In order to explore the potential clinical impact of our findings, we measured *LIF* expression levels by qRT-PCR in peripheral blood samples from 33 patients with CMPNs and 6 healthy controls. In agreement with our previous findings, *LIF* expression levels were significantly increased

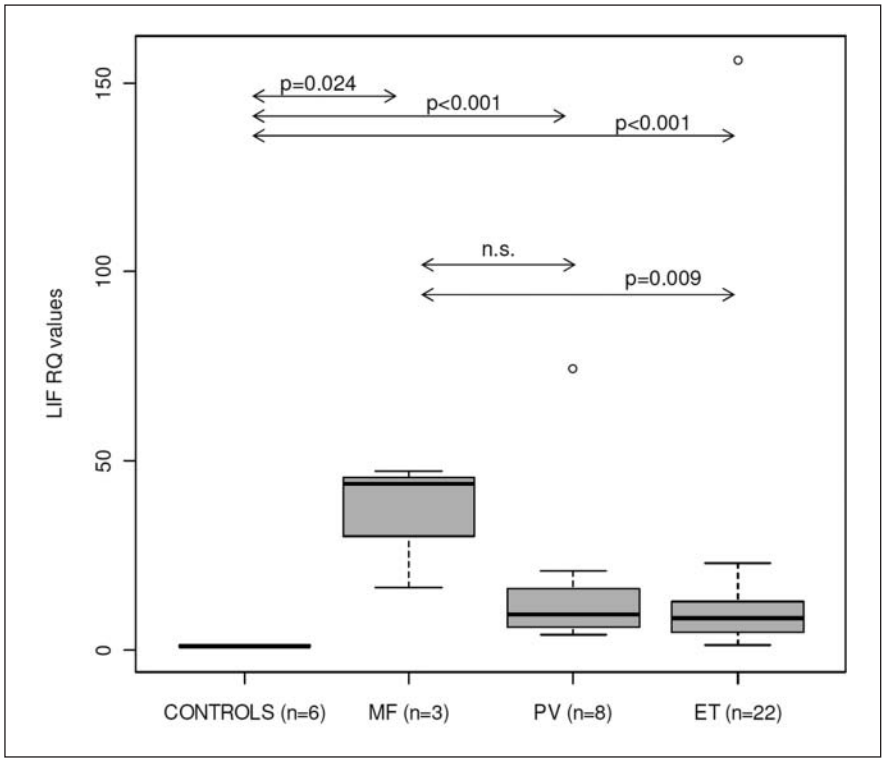

Figure 2. *LIF* expression is significantly increased in MPN patients. Relative quantification by real-time RT-PCR in peripheral blood samples from 6 healthy controls and 33 MPN patients (3 myelofibrosis, 8 polycytemia vera, and 22 essential thrombocytemia). Boxplots show the median (horizontal line), interquartile range (box), and range (whiskers). All results were normalized to the average of the 6 control samples. Significance of the Mann-Whitney test is shown for several comparisons.

in CMPN samples (Fig. 2). Furthermore, significant differences were found between idiopathic myelofibrosis (MF) and essential thrombocytemia (ET) samples. Because all patient samples had been analyzed for mutations in *JAK2* (V617F and exon 12 mutations) and in *MPL* (exon 10 mutations), we could also compare *LIF* expression levels between mutated and nonmutated samples. However, all polycytemia vera (PV) samples were mutated, and we only had 3 MF samples. Therefore, we could only compare mutated versus nonmutated ET samples, finding significantly higher *LIF* expression levels in ET mutated samples ($P = 0.048$, Mann-Whitney test). Interestingly, expression levels of *OSM*, a canonical STAT5 target gene, were also significantly increased in patient samples but did not discriminate between MF and ET patients or between mutated and nonmutated ET samples (not shown).

In conclusion, we show that *LIF* is directly regulated by STAT5 and that it is overexpressed in myeloid cell lines with increased activity of the JAK2/STAT5 signaling pathway. Furthermore, *LIF* expression is significantly increased in CMPN patients, particularly MF cases and samples with activating mutations in *JAK2* or in *MPL*. It is interesting that *LIF* codes for a cytokine that binds the heterodimeric gp130/LIFR receptor and phosphorylates STAT3. In this respect, *LIF* behaves like *OSM*, a STAT5-regulated gene coding for a cytokine whose receptor signals via STAT3. This, in murine leukemia cells, leads to increased expression of the receptor for IL-3,[7] which signals via JAK2/STAT5. Thus, *LIF* might represent a crucial link in the coordination of STAT3 and STAT5 signaling. Similarly, it could have a role in determining the balance between STAT5 and STAT1 activation, which has recently been proposed to be one of the major determinants of clinical phenotype in MPNs with mutations in *JAK2*.[8] The fact that *LIF* and *OSM* expression are increased in MPN samples negative for mutations in *JAK2* and *MPL* lends further support to the existence of additional genetic or epigenetic events that contribute to activate the JAK2/STAT5 pathway. Furthermore, *LIF* (but not *OSM*) seems to discriminate between MF and PV or ET patients, suggesting that effectors of JAK2/STAT5 signaling (such as STAT5-regulated genes) might explain the phenotypic variability observed in patients with constitutive activation of this signaling pathway. The finding that a multifunctional cytokine involved in the maintenance of pluripotency is a transcriptional target of STAT5 and is upregulated in MPNs might improve our understanding of the mechanisms governing STAT5-mediated oncogenesis.

Materials and Methods

Cell lines and patient samples. Binding of STAT5 and activation/inhibition studies were performed in cell lines where JAK2/STAT5 activity can be induced with IL-3 or in cell lines with constitutively active signaling that can be suppressed by specific inhibitors. We used M07e cells, a myeloid cell line strictly dependent on IL-3, the HEL cell line (human erythroleukemia cells that harbor the V617F activating mutation in *JAK2* gene), and SET-2 cells (a cell line heterozygous for the V617F mutation in *JAK2*, originally derived from a patient with ET at megakaryoblastic transformation). For stimulation studies, M07e cells were

starved of growth factors for 16 hours and then treated with 30 ng/mL IL-3 for 45 minutes. For inhibition experiments, cells were treated with 100 uM STAT5 inhibitor (cat. no. 573108, Calbiochem) for 1 hour.

LIF expression was also assayed in peripheral blood samples obtained (with informed consent) from 33 patients with CMPNs, all of whom were positive for the V617F mutation in *JAK2* (12 males and 21 females). Eight patients had the diagnosis of PV, 3 had MF, and 22 had ET.

Chromatin immunoprecipitation. Control cells (untreated) and cells in which JAK2/STAT5 signaling was induced with IL-3 were incubated with 1.5% formaldehyde (Fluka, St. Louis, MO), cell pellets were washed, and nuclear extracts were sonicated using a Bioruptor apparatus (Diagenode, Liege, Belgium) for 13 minutes (30-second pulses) on ice. Supernatants were incubated overnight with a polyclonal STAT5-specific antibody (C-17, Santa Cruz Biotechnology Inc., Santa Cruz, CA) under rocking conditions at 4°C, while an aliquot was set aside before inmunoprecipitation ("input" DNA sample). After incubation for 3 hours with protein A sepharose (GE Healthcare, Waukesha, WI), specific complexes were washed and recovered from beads with an extraction buffer. Finally cross-links were reversed, and both immunoprecipitated and input DNA samples were recovered by phenol:chloroform extraction, ethanol precipitation, and redissolved in nuclease-free water. DNA recovered from the procedure was amplified with GenomePlex WGA and purified with the GenElute PCR Clean-Up Kit (both from Sigma-Aldrich, St. Louis, MO). In order to quantify the enrichment of specific regions in IL-3–treated cells, relative to noninduced cells, immunoprecipitated and input DNA were used as templates in qPCR reactions using specific primers flanking putative STAT5-binding motifs in the promoter of *LIF*, with SYBR GreenER qPCR SuperMix Universal (Invitrogen, Paisley, UK) in an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The amount of PCR product in immunoprecipitated samples was normalized to input DNA in both experimental conditions (untreated or IL-3–treated M07e cells).

Quantification of gene expression. For *LIF* qRT-PCR assays, total RNA was isolated with TRIzol reagent (Invitrogen) and quantitated using a NanoDrop spectrophotometer (NanoDrop Technologies, Wilmington, DE). One microgram of total RNA was converted to cDNA using SuperScript III First-Strand Synthesis SuperMix (Invitrogen), and 25 ng of cDNA was amplified using specific primers (LIF_F: 5′-CAACTGGCACAGCTCAATGG-3′ and LIF_R: 5′-CTCCCCCTGGGCTGTGTA-3′) with SYBR GreenER qPCR SuperMix Universal (Invitrogen) in an ABI 7500 Real-Time PCR System (Applied Biosystems). As endogenous control, we used *TBP*, a housekeeping gene that showed consistent expression levels across different experimental conditions.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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