

Brief report

Murine embryonic stem cell differentiation is promoted by SOCS-3 and inhibited by the zinc finger transcription factor Klf4

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Embryonic stem (ES) cells homozygous for a Shp-2 mutation (Shp-2^{Δ46-110}) demonstrate leukemia inhibitory factor (LIF) hypersensitivity and increased LIF-stimulated phosphorylation of signal transducer and activator of transcription (STAT3). We hypothesized that LIF-responsive genes in Shp-2^{Δ46-110} cells would represent potential candidates for molecules vital for ES cell self-renewal. Using microarray analysis, we detected 41 genes whose expres-

sion was modified by LIF in Shp-2^{Δ46-110} ES cells. Induction of 2 significantly up-regulated genes, suppressor of cytokine signaling-3 (SOCS-3) and Krüppel-like factor 4 (Klf4), was verified using Northern blotting. ES cells overexpressing SOCS-3 had an increased capacity to differentiate to hematopoietic progenitors, rather than to self-renew. In contrast, ES cells overexpressing Klf4 had a greater capacity to self-renew based on

secondary embryoid body (EB) formation. Klf4-transduced d6 EBs expressed higher levels of Oct-4, consistent with the notion that Klf4 promotes ES cell self-renewal. These findings verify the negative role of SOCS-3 on LIF signaling and provide a novel role for Klf4 in ES cell function. (Blood. 2005;105:635-637)

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Introduction

The mechanisms that control self-renewal of somatic and embryonic stem (ES) cells are unknown; however, identification of regulating molecules is essential for developing stem cell-based therapeutic strategies. Leukemia inhibitory factor (LIF) promotes murine ES cell self-renewal and maintains pluripotency in culture.¹ LIF binds the heterodimeric LIF receptor-glycoprotein 130 (gp130) complex and activates the Jak kinases with recruitment of Shp-2 and signal transducer and activator of transcription (STAT3).² Mutation of STAT3-interacting tyrosines on gp130 reduces LIF-stimulated phospho-STAT3 and STAT3-DNA-binding activity as well as ES cell self-renewal, providing strong evidence that LIF-stimulated STAT3 activation is critical for ES cell self-renewal.^{3,4} Mutation of the Shp-2-binding tyrosine on gp130 prolongs STAT3 activation and increases self-renewal, whereas it diminishes Erk activation, indicating that Shp-2 functions to balance LIF-stimulated STAT3 and Erk activation, thus modulating ES cell self-renewal and differentiation, respectively.⁵

Shp-2^{Δ46-110} ES cells, which are homozygous for an exon 3 deletion from the murine *SHP-2* locus,⁶ have increased sensitivity to LIF and increased self-renewal capacity as assayed by secondary embryoid body (EB) formation, both of which are normalized upon reintroduction of wild-type (WT) Shp-2.^{7,8} Consistently, Shp-2^{Δ46-110} ES cells manifest increased LIF-stimulated phospho-STAT3 and decreased LIF-stimulated phospho-Erk activity.⁷

Because LIF-stimulated STAT3 is crucial for ES cell self-renewal, we hypothesized that LIF-induced genes would be

fundamental in ES cell self-renewal. Additionally, because LIF-induced phospho-STAT3 levels are higher in Shp-2^{Δ46-110} cells than in WT cells,⁷ we reasoned that LIF stimulation of Shp-2^{Δ46-110} cells would provide a sensitive system for the detection of STAT3-regulated genes. Therefore, we identified LIF-regulated genes by comparing expression profiles of Shp-2^{Δ46-110} ES cells either unstimulated or stimulated with LIF. The cDNAs for 2 LIF-stimulated genes, which encode suppressor of cytokine signaling-3 (SOCS-3) and the transcription factor, Krüppel-like factor 4 (Klf4), were introduced into WT ES cells for functional analysis.

Study design

ES cell culture and microarray analysis

The Shp-2^{Δ46-110} cell line, IC3, was generated as described.⁷ IC3 cells were cultured in serum-free, LIF-free media for 6 hours followed by treatment with phosphate-buffered saline (PBS) or LIF (1000 U/mL) for 45 minutes. Total RNA was extracted using QIAamp RNA Mini kit (Qiagen, Valencia, CA) and submitted to the Center for Medical Genomics at the Indiana University School of Medicine for labeling and hybridization to Affymetrix Murine Genome U74A GeneChips (Affymetrix, Santa Clara, CA). LIF stimulation, RNA preparation, and microarray analysis were performed in triplicate and analyzed with MAS5 software (Affymetrix, Santa Clara, CA). The *t* tests using the signal were performed using CyberT⁹ for genes present in at least one half of the arrays for either condition.¹⁰ Confirmatory Northern blots were conducted as described.¹¹

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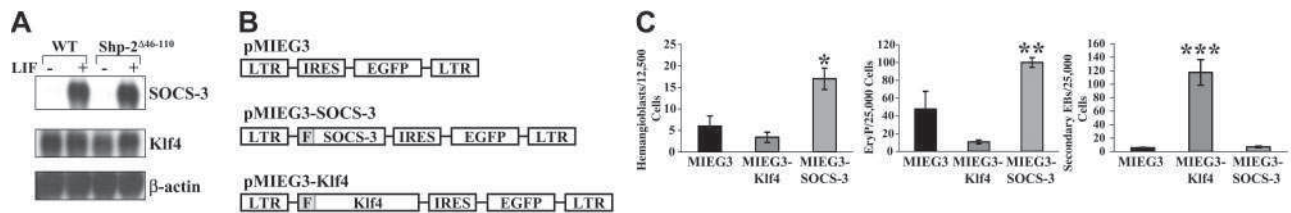


Figure 1. SOCS-3 promotes, whereas Klf4 inhibits, ES cell differentiation. (A) Northern blot analysis of LIF stimulation of *SOCS3* and *KLF4* in WT and Shp-2^{Δ46-110} ES cells (representative of 2 independent experiments). (B) Schematic diagram of retroviral vectors used to conduct functional studies. IRES indicates internal ribosome entry site; LTR, long terminal repeat. (C) Transduced and sorted ES cells were subjected to in vitro differentiation for hemangioblasts, primitive erythroid (Ery^P) progenitors, and secondary EBs; data represent 2 independent experiments with cultures plated in triplicate; **P* = .02 for pMIEG3-SOCS-3 versus pMIEG3; ***P* = .03 for pMIEG3-SOCS-3 versus pMIEG3; ****P* = .0002 for pMIEG3-Klf4 versus pMIEG3. Error bars represent SEM. Statistics were analyzed using the 2-tailed, unpaired, Student *t* test.

Retroviral plasmids, ES cell transduction, and in vitro differentiation

The *SOCS-3* cDNA and the *Klf4* cDNA (from Dr Vincent Yang, Emory University School of Medicine, Atlanta, GA) were epitope-tagged with FLAG at the 5' end, subcloned into the retroviral vector, pMIEG3, in tandem with enhanced green fluorescent protein (EGFP), and confirmed by sequencing. Retroviral supernatants were prepared using Eco-Phoenix packaging cells by the Indiana University Vector Production Facility.

R1 (WT) ES cells were transduced using polybrene (40 mg/mL) over 48 hours. EGFP⁺ cells were collected using fluorescence-activated cell sorting and subjected to hematopoietic in vitro differentiation assays, as described.^{7,12} Transduction, sorting, and in vitro differentiation assays were conducted in 2 independent experiments.

Immunoprecipitation and immunoblotting

Proteins from EGFP⁺ cells were immunoprecipitated as described¹³ and probed with anti-SOCS-3, anti-Klf4 (Santa Cruz Biotechnology, Santa Cruz, CA), or anti-FLAG (Sigma, St Louis, MO). For Oct-4 detection, 50 μg protein extract was used and probed with anti-Oct-4 (Santa Cruz Biotechnology) or with anti-β-actin to control for protein loading. Signals were detected by enhanced chemiluminescence and quantitated using ImageJ (National Institutes of Health, Bethesda, MD).

Results and discussion

To define an appropriate time to evaluate LIF-stimulated gene expression, we determined that *c-fos* mRNA induction, known to be regulated by LIF-induced STAT3 activation,^{14,15} was maximal following LIF treatment for 45 minutes (not shown). Total cellular RNA from Shp-2^{Δ46-110} ES cells treated with PBS or LIF 1000 U/mL for 45 minutes was submitted for microarray analysis, which revealed that 41 genes or expressed sequence tags were significantly modified (*P* < .01, determined by *t* test on the signal value, Tables S1 and S2; see the Supplemental Tables link at the top of the online article link at the top of the online article on the *Blood* website). The 2 most significantly up-regulated genes were *SOCS3* (increase 34.4-fold; *P* < .000001) and *KLF4* (increase 2.1-fold; *P* = .000057). Using Northern blot, we examined the effect of LIF on *SOCS3* and *KLF4* expression in both WT (R1) and Shp-2^{Δ46-110} cells. In the Shp-2^{Δ46-110} cells, *SOCS3* expression was increased dramatically and *KLF4* expression was increased approximately 2-fold, in agreement with the microarray results (Figure 1A). *SOCS3* was similarly induced in the WT cells. However, at baseline, *KLF4* expression was elevated in the WT ES cells compared to the Shp-2^{Δ46-110} cells and was not induced by LIF treatment (Figure 1A).

To examine *SOCS-3* and *Klf4* function within ES cells, each cDNA was introduced into R1 ES cells using retroviral transduction (Figure 1B). Overexpression of *SOCS-3* promoted ES cell

differentiation to both hemangioblasts and primitive erythroid progenitors, whereas overexpression of *Klf4* demonstrated a trend toward reduced differentiation (Figure 1C). *Klf4*-transduced ES cells retained a high capacity to generate secondary EBs, suggesting that *Klf4* promotes ES cell self-renewal in addition to inhibiting ES cell differentiation (Figure 1C). These data support previous studies indicating that *SOCS-3* acts as a negative regulator of LIF signaling¹⁵⁻¹⁷ and provide a novel role for *Klf4* in ES cell differentiation and self-renewal.

Based on the differences in ES cell function induced by *SOCS-3* or *Klf4* overexpression, we determined the exogenously introduced and total *SOCS-3* and *Klf4* protein levels achieved in the transduced ES cells. Immunoblotting with anti-FLAG demonstrated that the exogenously introduced *SOCS-3* and *Klf4* were expressed in the transduced ES cells (Figure 2A). Densitometric measurements demonstrated a 3-fold increase in total *SOCS-3* protein and a 6-fold increase in total *Klf4* protein compared to cells transduced with vector alone (Figure 2A).

Because the secondary EB assay suggested that *Klf4* promotes ES cell self-renewal, we examined the level of Oct-4 protein, which is essential for ES cell self-renewal,^{18,19} in day-6 EBs. We observed that Oct-4 was significantly elevated (7-fold) in the *Klf4*-transduced d6 EBs compared to that of vector alone or *SOCS-3*-transduced d6 EBs (Figure 2B). These results support a role for *Klf4* in modulating ES cell self-renewal.

This study has defined a battery of LIF-sensitive genes within murine ES cells that provide potential mediators of ES cell

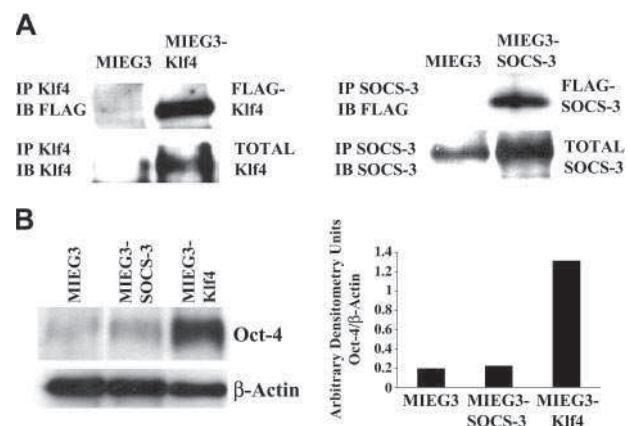


Figure 2. Overexpression of Klf4 results in sustained Oct-4 expression. (A) Immunoprecipitation studies demonstrating that the exogenously introduced (FLAG epitope-tagged) *Klf4* and *SOCS-3* proteins are expressed in transduced ES cells and that, consistently, total *Klf4* and *SOCS-3* protein levels are increased compared to cells transduced with vector alone. (B) Evaluation of Oct-4 protein levels in d6 EBs derived from pMIEG3-, pMIEG3-SOCS-3-, and pMIEG3-Klf4-transduced ES cells. Oct-4 band intensities normalized to β-actin band intensities are shown graphically. IP indicates immunoprecipitation; IB, immunoblotting.

self-renewal and differentiation. Functional evaluation demonstrated a novel role for Klf4 in ES cell differentiation and self-renewal. Klf4 is abundant in epithelial cells of the intestine, colon, skin, and thymus²⁰ and has been implicated as a negative prognosticator in breast cancer^{21,22} and as a tumor suppressor gene in gastrointestinal malignancies.^{23,24} Based on the differential effect of LIF-stimulated *KLF4* expression in the WT and Shp-2^{Δ46-110} cells, it is possible that altered Klf4 expression partially accounts for the increased self-renewal phenotype in the Shp-2^{Δ46-110} cells. In contrast, we observed that SOCS-3 promotes ES cell differentiation, likely by diminishing the self-renewing effects of LIF. Our findings support the conclusions of others that SOCS-3 is regulated at the transcriptional level by LIF^{17,25} and functions as a feedback

inhibitor on LIF signaling.^{16,17} These data provide feasible candidate genes for ES cell self-renewal, support a negative role of SOCS-3 on LIF signaling in ES cells, and present a novel role for Klf4 in ES cell function.

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