HEMATOPOIESIS AND STEM CELLS

Posttranscriptional regulation of c-Myc expression in adult murine HSCs during homeostasis and interferon-α-induced stress response

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Key Points

- Increasing levels of c-Myc protein during differentiation of HSCs are posttranscriptionally regulated by the ubiquitin-proteasome system.
- Interferon-α-mediated entry of quiescent HSCs into the cell cycle is associated with robust upregulation of c-Myc protein but not mRNA.

Previous studies have established pivotal roles for c-Myc and its homolog N-Myc in hematopoietic stem cell (HSC) maintenance and niche-dependent differentiation. However, it remains largely unclear how c-Myc expression is regulated in this context. Here, we show that HSCs and more committed progenitors express similar levels of c-myc transcripts. Using knock-in mice expressing a functional enhanced green fluorescent protein-c-Myc fusion protein under control of the endogenous *c-myc* locus, c-Myc protein levels were assessed. Although HSCs express low levels of c-Myc protein, its expression increases steadily during progenitor differentiation. Thus, mRNA and protein expression patterns differ significantly in stem/progenitor cells, suggesting that c-Myc expression is largely controlled posttranscriptionally. Moreover, interferon-α exposure, which activates dormant HSCs, strongly induces c-Myc expression at the protein level but not at the transcript level. This posttranscriptional mechanism of c-Myc regulation provides the blood system with a rapid way to adjust c-Myc expression according to demand during hematopoietic stress. (*Blood*. 2014;123(25):3909-3913)

Introduction

The hematopoietic stem cell (HSC) pool is maintained by self-renewal while giving rise to mature cell types of our blood system by differentiation. Previous work by our group has shown that c-Myc controls this delicate balance between HSC self-renewal and differentiation.[1,2] Although c-Myc overexpression leads to loss of functional HSCs in vivo, c-Myc-deficient HSCs in conditional knock-out mice accumulated in the bone marrow (BM), most likely because of failure to exit the niche and differentiate. Consequently, mice suffered from pancytopenia and died after 6 to 8 weeks. Concurrent induced deletion of c-myc and N-myc in the BM of mice resulted in rapid and severe pancytopenia that culminated in lethality as early as 12 days after Cre induction.[3] However, little is known about how Myc activity, and consequently its crucial function, are regulated in HSCs. Here we investigated the regulation of c-Myc expression at the transcriptional and posttranscriptional level in HSCs during homeostasis and stress.

Study design

Animal experiments

Procedures were approved by the Regierungspräsidium Karlsruhe (G-127/08 and G-145/11). Mice used in experiments were 7 to 12 weeks old. Enhanced green fluorescent protein (eGFP)-c-Myc knock-in mice ($Myc^{tm1Slek}$) were provided by Barry Sleckman.[4] For polyI:C treatment, mice were injected intraperitoneally with 100 μg/mouse (Invivogen, San Diego, CA) in phosphate-buffered saline 72 and 24 hours prior to analysis. Control mice were C57BL/6JOlaHsd (Harlan, Boxmeer, Netherlands).

Cell culture and treatment

Lin^- BM cells were isolated and cultured in StemPro-34 with nutrients (Gibco, Carlsbad, CA) in the presence of thrombopoietin (TPO), stem cell factor, and Fms-related tyrosine kinse 3 ligand (Flt3L) (R&D Systems, Minneapolis, MN). Cells were incubated with either dimethylsulfoxide (Sigma, Schnelldorf, Germany) as a control or 10 μM MG-132 (Calbiochem, San Diego, CA) and/or 50 μg/mL cycloheximide (Th. Geyer, Renningen, Germany).

Flow cytometry

BM was isolated and lineage depleted as previously described.[3] For an overview of antibodies used and cell surface phenotypes, see supplemental Tables 1 and 2 available on the *Blood* Web site. Dead cells were excluded with 7-Aminoactinomycin (7-AAD) (Becton Dickinson, San Jose, CA). Analyses were performed on LSRII/Fortessa (Becton Dickinson). Cell sorting was performed on a fluorescence-activated cell sorter (FACS) AriaI/II (Becton Dickinson). Data were analyzed with FlowJo software (Tree Star, Ashland, OR).

RNA isolation and real-time polymerase chain reaction

A total of 1000 to 20 000 cells were sorted in Extraction Buffer, and RNA isolation was performed according to the PicoPure RNA isolation kit

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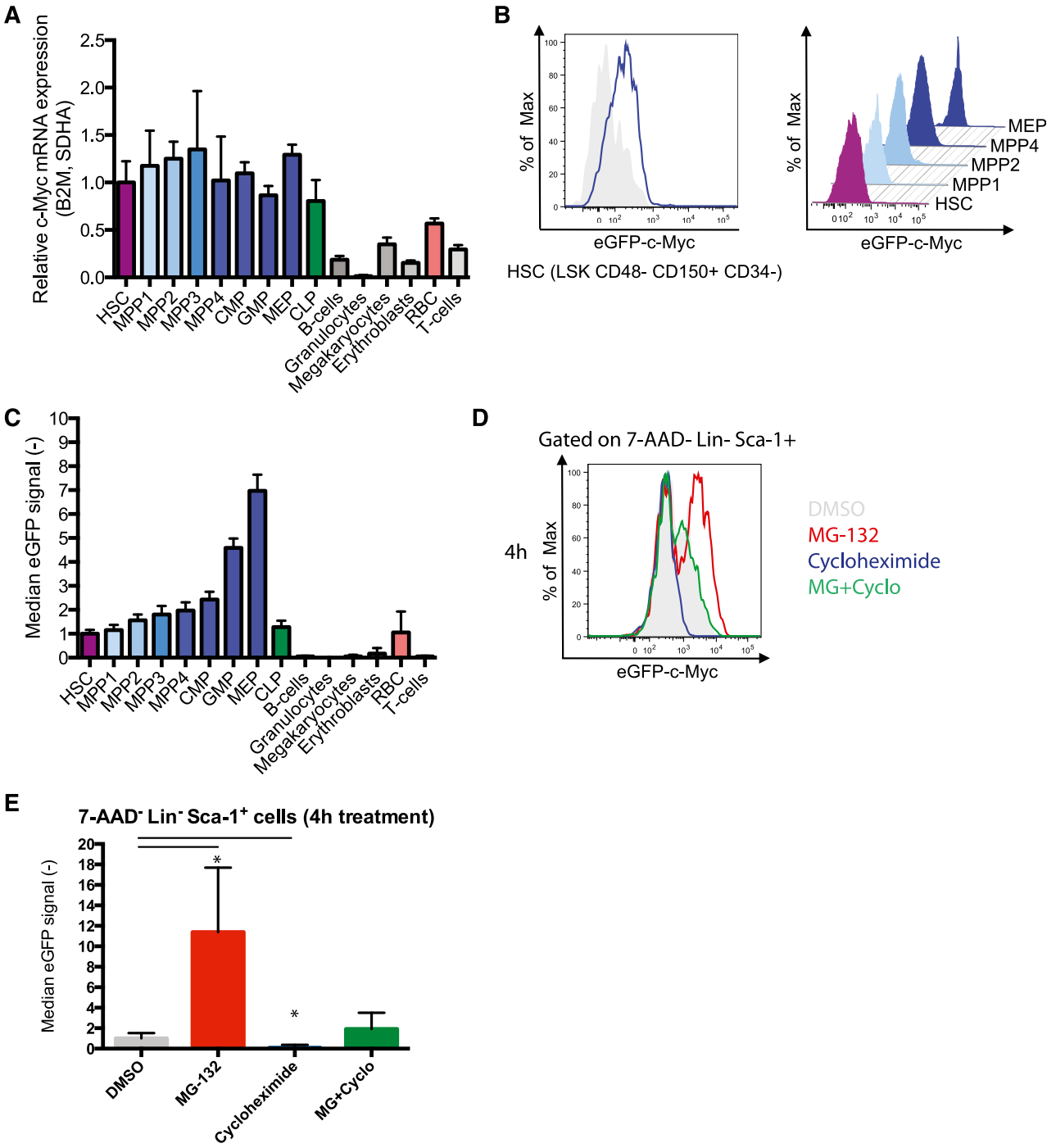

Figure 1. Expression of c-Myc in the hematopoietic system is regulated by posttranscriptional mechanisms. (A) mRNA expression of c-Myc by hematopoietic cells of the bone marrow were measured by qRT-PCR (pooled data of 2 independent experiments; N = 8; mean and standard deviation (SD); no significant differences between HSCs and progenitors according to 1-way analysis of variance followed by Dunnett's multiple comparison test). (B) Histogram of eGFP-c-Myc fluorescence of HSCs and overlay of eGFP-c-Myc fluorescence of HSCs, multipotent progenitor 1 (MPP1), MPP2, MPP4, and MEPs. The eGFP signal is shown in blue, and the background signal of control cells is in gray. (C) eGFP-c-Myc protein expression of hematopoietic cells of the bone marrow as measured by flow cytometry, expressed as background-corrected median eGFP fluorescence (pooled data of 3 independent experiments; N = 12; mean and SD; 1-way analysis of variance followed by Dunnett's multiple comparison test: differences between HSCs and MPP2/MPP3/MPP4/common myeloid progenitors [CMP]/granulocyte-macrophage progenitors [GMP]/megacaryocyte-erythroid progenitors [MEP] are significant). (D) eGFP-c-Myc expression of Lin^- cells cultured in vitro in the presence or absence of proteasome inhibitor MG-132 and/or translation inhibitor cycloheximide as measured by flow cytometry. (E) Quantification of D. Plotted are background-corrected median eGFP signals (pooled data of 2 independent experiments; N = 8; $^*P < .05$; unpaired 2-tailed *t* test). For a list of abbreviations of hematopoietic cell types and markers used to identify them, refer to Supplemental Table 1.

manual (Life Technologies, Carlsbad, CA) including RNase-free DNase digestion (Qiagen, Hilden, Germany). RNA samples were transcribed using the SuperScript VILO cDNA synthesis kit using additional oligo-dT primers (Life Technologies). Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed using the ABI Power SYBR Green Master Mix (Life Technologies). PCR reactions were performed on a Viia7 (Life Technologies). Primers are listed in supplemental Table 3.

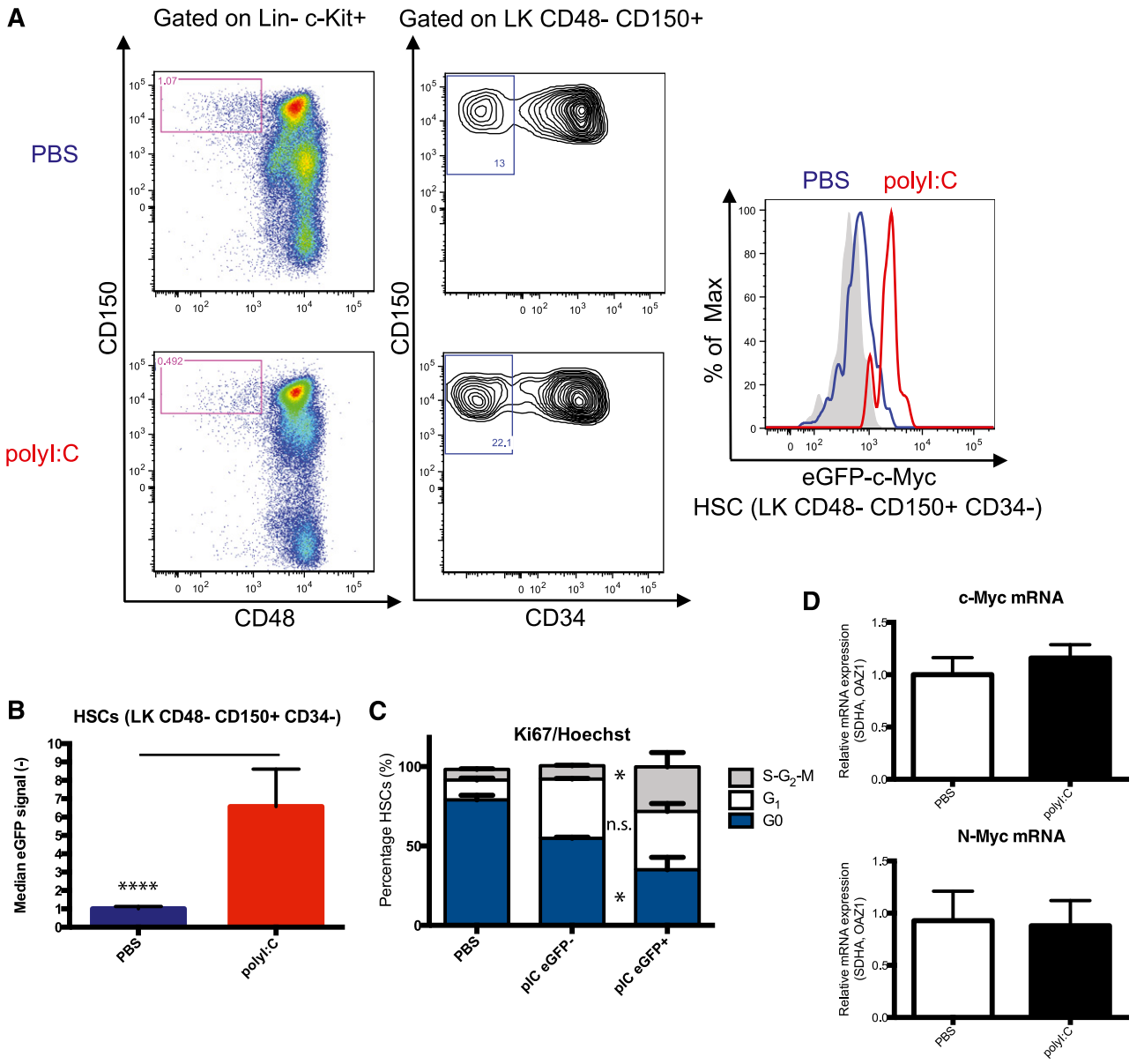

Figure 2. eGFP-c-Myc protein expression is induced by polyI:C and IFN-α in HSCs. (A) Treatment of eGFP-c-Myc knock-in mice with polyI:C induces eGFP-c-Myc expression in HSCs as shown by flow cytometry. Sca-1 was omitted as a marker, as polyI:C leads to a shift in Sca-1 expression. (B) Quantification of A (pooled data of 3 independent experiments; N = 12; mean and SD; ****$P < .001$; unpaired 2-tailed *t* test). (C) Correlation of cell cycle status (Ki67-Hoechst) and eGFP-c-Myc expression of HSCs after polyI:C treatment (N = 3; mean and SD; *$P < .05$; unpaired 2-tailed *t* test). (D) mRNA expression of c-Myc and N-Myc were measured by qRT-PCR in FACS-sorted $Lin^-Kit^+CD48^-CD150^+$ cells of wild-type mice injected with phosphate-buffered saline or polyI:C (pooled data of 2 independent experiments; N = 6-8 per group; mean and SD; unpaired 2-tailed *t* test).

Results and discussion

c-Myc activity controls the delicate balance between HSC self-renewal and differentiation,[1,2] and it therefore is of great importance to understand how the expression of *c-myc* is regulated in this context. First, we determined *c-myc* mRNA expression in several FACS-sorted hematopoietic populations in the BM by qRT-PCR. We observed that *c-myc* mRNA expression levels in HSCs and more committed progenitors are comparable (Figure 1A). However, protein levels, rather than mRNA levels, are critical for its activity. To measure the low expressed c-Myc protein in hematopoietic subpopulations, we took advantage of the eGFP-cMyc knock-in mouse model.[4] Using flow cytometry, different hematopoietic cell populations of homozygous eGFP-cMyc knock-in mice were immunophenotypically identified, which enabled us to measure eGFP-c-Myc expression at the single cell level. This analysis revealed that median eGFP-c-Myc protein expression is low in HSCs, as expected, but increases steadily during early hematopoietic progenitor differentiation, culminating in megakaryocyte-erythroid progenitors (Figure 1B-C). This result is consistent with previous genetic data showing that c-Myc deficient HSCs fail to leave the BM niche and supports the resulting hypothesis that c-Myc upregulation is necessary for initiating HSC differentiation and subsequent progenitor proliferation.[1,2] Terminally differentiated cell types in the BM, except for red blood cells, show relatively low c-Myc protein expression (Figure 1C). Because *c-myc* transcript levels remain rather constant during the maturation of HSCs toward committed progenitors

while c-Myc protein steadily increases, the data strongly suggest that during this process, c-Myc expression is regulated posttranscriptionally. To confirm that c-Myc expression is controlled by posttranscriptional mechanisms, we cultured Lin^- cells in the presence or absence of the proteasome inhibitor MG-132 and/or the translation inhibitor cycloheximide. Although inhibition of the proteasome led to rapid accumulation of eGFP-c-Myc protein in Lin^-Sca1^+ cells, translational inhibition resulted in the rapid loss of eGFP-c-Myc expression (Figure 1D-E; supplemental Figure 1), suggesting that c-Myc protein levels in immature hematopoietic cells are controlled by de novo translation and degradation through the ubiquitin-proteasome system. By this mechanism, there is potential for upregulation of c-Myc protein expression in HSCs without an increase in transcription, which might allow for a rapid reaction to injury signals that activate proliferation and differentiation programs, such as in the context of bacterial or viral infections or after toxic insults such as chemotherapy.

As c-Myc activity promotes metabolic growth[5,6] and proliferation,[7] we hypothesized that stress-activated HSCs may express higher c-Myc levels. We and others previously demonstrated that interferon-α (IFN-α) is capable of activating dormant HSCs and driving them into the cell cycle.[8,9] Hence, we asked whether IFN-α-induced proliferation is associated with c-Myc induction. To test this hypothesis, we injected eGFP-cMyc knock-in mice[4] intraperitoneally with polyI:C, which induces IFN-α production. Indeed, polyI:C stimulation in vivo induced a 6.6-fold increase (standard deviation, ± 2.0) in eGFP-cMyc expression in $Lin^-Kit^+CD150^+CD48^-CD34^-$ HSCs as measured by flow cytometry, which correlated with their cell cycle status (Figure 2A-C). Sca-1 was omitted as a surface marker of HSCs, because it is itself strongly upregulated in response to IFN-α stimulation.[9,10] Similarly, induction of eGFP-cMyc expression in HSCs could also be observed after subcutaneous injection of IFN-α (data not shown). Interestingly, *c-myc* and *N-myc* mRNA levels remain unchanged in HSCs in response to polyI:C treatment, suggesting that c-Myc protein is induced posttranscriptionally in response to this stress signal (Figure 2C). *L-myc* expression was neither detectable before nor after polyI:C treatment (data not shown). In summary, c-Myc protein expression in HSCs is rapidly induced posttranscriptionally in response to IFN-α. Together with the known proliferative function of c-Myc, this suggests that c-Myc may play a functional role in IFN-α-induced HSC activation.

Transcription factors regulating *c-myc* mRNA expression have attracted recent attention,[11] but it is now emerging that posttranscriptional mechanisms, for instance, E3 ubiquitin ligases, regulate tissue- and cell type-specific c-Myc activity. Thus, we analyzed the mRNA expression of several candidate E3 ubiquitin ligases in different HSC and progenitor compartments. Our data suggest that several of these ligases may regulate c-Myc levels, of which Trim32 and Trpc4ap exhibit mRNA patterns that negatively correlate with c-Myc protein levels at homeostasis and after polyI:C (supplemental Figures 2-4).

Interestingly, it was recently demonstrated that Fbw7 controls c-Myc protein stability in HSCs,[12] suggesting that c-Myc expression in HSCs is regulated to a large extent on the posttranscriptional level and that Fbw7 is one of the negative posttranscriptional regulators involved. Our data provide new insights into the regulation of c-Myc protein expression in HSCs in homeostasis and the IFN-α-induced stress response. This is likely to be important beyond the scope of normal stem cell biology, because c-Myc is critical in other stem cells and is one of the most potent oncogenes frequently deregulated in hematologic and solid malignancies.[13-17]

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Authorship

Contribution: A.E., M.A.E., and A.T. designed the research; A.E., T.B., M.A.E., H.U., and K.M. performed experiments and analyzed the data; B.P.S. generated and provided eGFP-c-Myc knock-in mice; and A.E. and A.T. wrote the manuscript.

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