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Comparative Long-term Effects of Interferon Alpha and Hydroxyurea on Human Hematopoietic Progenitor Cells

Katherine Y. King^{1,3,*}, Katie A. Matatal^{1,3}, Ching-Chieh Shen², Margaret A. Goodell³, Sabina I. Swierczek⁴, and Josef T. Prchal⁴

¹ Department of Pediatric Infectious Diseases, Baylor College of Medicine, Houston, Texas

² School of Medicine, Washington University, St. Louis, Missouri

³ Stem Cells and Regenerative Medicine Center, Baylor College of Medicine, Houston, Texas

⁴ Department of Hematology, University of Utah School of Medicine, Salt Lake City, Utah

Abstract

Interferon alpha (IFN α) is used clinically to restore polyclonal hematopoiesis in patients with the myeloproliferative neoplasms (MPN) polycythemia vera (PV) and essential thrombocythemia (ET), and to improve chemosensitivity in chronic myeloid leukemia patients. However, the mechanisms by which IFN α affects disease-initiating hematopoietic stem and progenitor cells (HSPCs) remain poorly understood. While IFN α has been shown to transiently impair quiescence of murine hematopoietic stem cells, its effects on human HSPCs have not been studied *in vivo*. Here, we compared bone marrow serially obtained from MPN patients before and during pegylated IFN α (Peg-IFN α) treatment against marrow serially obtained from patients on hydroxyurea. The percentage of HSPCs actively undergoing cell cycle was increased after Peg-IFN α treatment in a majority of patients compared to hydroxyurea-treated controls, suggesting that IFN α promotes cell division. Furthermore, transcriptional profiling revealed that cell cycle-associated genes were induced, while genes involved in HSPC quiescence were repressed during IFN α treatment. Compared to hydroxyurea-treated controls, Peg-IFN α treated patients had similar HSPC numbers but increased numbers of hematopoietic progenitors as determined by colony formation assay, indicating an increase in myeloid proliferation/differentiation. These effects occurred regardless of JAK2 mutational status. Together, these data provide the first *in vivo* evidence that Peg-IFN α promotes cell division and differentiation of human HSPCs.

*Corresponding Author Katherine Y. King MD PhD Feigin Center for Pediatric Research Texas Children's Hospital 1102 Bates Street Suite 1150 Houston, TX 77030 kyk@bcm.edu Phone 832-824-4330 Fax 832-825-4347.

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Authorship Contributions and Disclosures

KAM and CCS performed experiments and data analysis. SIS performed experiments. MAG assisted in conceptualization and study design and edited the paper. JTP enrolled patients in the study. designed the study. and edited the paper. KYK designed the study. performed experiments. conducted data analysis. and wrote the paper. The authors report no potential conflicts of interest.

Introduction

Interferon alpha (IFN α) is used in a variety of clinical settings, including treatment of viral infections (hepatitis C, hepatitis B, human herpesvirus 8), multiple sclerosis, cancers (renal cell carcinoma, malignant melanoma) and hematological malignancies (hairy cell leukemia, polycythemia vera (PV) and essential thrombocythemia (ET), and chronic myelogenous leukemia)(1). For patients with PV and ET, IFN α remains the only drug that leads to restoration of polyclonal hematopoiesis(2) and, in a few cases, a possible cure(3). IFN α has also been shown to improve outcomes as an adjunctive therapy for patients with chronic myeloid leukemia(4). Furthermore, pegylated forms of IFN α (Peg-IFN α) improve the safety and tolerability of the drug, decreasing the frequency of its administration and changing its mode of administration from intravenous to subcutaneous, thereby making it more attractive and practical to use in many patients.

Despite its broad use in clinical settings, the mechanism by which IFN α affects hematopoietic stem and progenitor cells (HSPCs) remains poorly understood. While *in vitro* experiments suggest that IFN α has an inhibitory effect on the growth of human hematopoietic progenitor cells and may induce apoptosis(5-7), recent *in vivo* experiments in mice show that interferons promote cell division and increased differentiation of hematopoietic stem cells (HSCs)(8). Differentiation comes at the expense of self-renewal and can ultimately lead to exhaustion of the HSC compartment(8-10). A recent study further showed that the stressful effects of *in vitro* culture can dramatically alter experimental results after interferon exposure, indicating that *in vitro* studies do not effectively model clinical conditions (11). These studies highlight the need to examine interferon responses by human HSPCs in an *in vivo* setting.

PV and ET are clonal disorders of hematopoiesis thought to arise from the level of the HSC (12). Both IFN α and hydroxyurea are known to induce hematological remission of these disorders, but their mechanisms of action may be quite different. Here, we characterized HSPCs consecutively obtained from patients with MPNs before and during treatment with either Peg-IFN α or hydroxyurea. We find that Peg-IFN α promotes cell division of HSPCs without altering their total number, in contrast to the cell cycle suppressive effects of the antimetabolite hydroxyurea. Furthermore, Peg-IFN α promotes myeloid colony formation by HSPCs and is associated with decreased expression of quiescence-associated genes such as MECOM (MDS1-EVI1) and MPL. Although MPNs are frequently associated with activating mutations in JAK2 (13), our findings were independent of JAK2 status; and the study was not designed to investigate differential effects of Peg-IFN α on WT versus JAK2-mutant clones. Rather, our data represent the first *in vivo* evidence that Peg-IFN α promotes cell division and terminal differentiation of human HSPCs in general. These findings provide insight into the mechanism of IFN α action on human HSPCs, information that is essential to guide future rational use of IFN α in a variety of clinical contexts.

Methods

Study design

This study included PV and ET patients who participated in two trials as a part of the Myeloproliferative Disorders Research Consortium (MPD) (n=22) and subjects treated off protocol (n=14). In both protocols, enrolled patients were randomized to either a dose escalation trial of Peg-IFN α given as four weekly subcutaneous injections of 45 micrograms, with dose escalation to 60, 90, 135 and 180 micrograms (the final dose was determined upon achieving complete hematological remission), or hydroxyurea treatment arms. Patient consent was obtained and all procedures were approved by the institutional review boards for the University of Utah School of Medicine and Baylor College of Medicine. Study subjects had at least two bone marrow biopsies, one pre-treatment and one or more during the course of treatment. Two subjects had two biopsies performed pre-treatment; these were used as “untreated controls.”

Bone marrow processing

Bone marrow samples were shipped overnight on ice and processed immediately upon receipt the next day. Samples were Ficoll separated using Lymphoprep (Stemcell Technologies) according to the manufacturer's protocol. Serum was immediately frozen at -80°C . The mononuclear layer was enriched for CD34+ cells using magnetic microbead separation (autoMACS; Miltenyi Biotec).

Interferon alpha levels

IFN α serum levels were tested using the VeriKine human IFN α serum sample ELISA Kit (PBL Interferon Source) according to the manufacturer's protocol.

Myeloid differentiation assay

Ficoll separated mononuclear cells were plated at a density of 1×10^5 per 2 mL methylcellulose with cytokines to support the growth of human myeloid progenitor cells (StemCell Technologies). Colonies were counted after 14 days and identified by morphology.

Flow Cytometry

Flow cytometric analysis was performed on an LSRII instrument (BD Biosciences). Hematopoietic progenitor cells were identified as size-selected CD45+ CD34+ CD38 $-$, unless otherwise stated. Antibodies used were from Miltenyi Biotec (CD45 clone 5B1; CD34 clone AC136; CD38 clone EPR4106). The cell cycle profile of samples was determined using Hoechst/Pyronin Y staining on CD34-enriched cells as previously described(14).

RNA isolation and real-time PCR

RNA was isolated from CD34+ cells using RNAqueous Total RNA Isolation Kit (Life Technologies). RNA was reverse transcribed using SuperScript II (Life Technologies) and preamplified 18-20 cycles using TaqMan PreAmp Master Mix and TaqMan probes (Life

Technologies). Real-time PCR was performed using TaqMan probes on a 48.48 Fluidigm Dynamic Array integrated fluidic circuit chip on the BioMark HD system (Fluidigm). Samples were run in triplicate and normalized to the geometric mean of three internal controls (*GAPDH*, *18S*, and *ACTB*). Gene expression for each treated sample was compared to the same individual's pre-treatment baseline expression for each gene of interest and shown as the log2 fold change over the baseline expression for that gene, unless otherwise stated. Data include more than one post-treatment sample from patients when available.

Quantification of the JAK2 allele

The *JAK2*^{V617F} mutational burden in gDNA purified from granulocytes or separated CD34+ cells was determined by quantitative allele-specific PCR (qRT-AS-PCR) as previously described(15, 16).

Statistics

Mean values $\pm$ SEM are shown. Unpaired two-tailed Student's t-test was used for all comparisons and Pearson's correlation coefficient was used to determine the significance of the linear correlation between the fold change in % S/G2/M phase of the cell cycle and *JAK2*^{V617F} mutational status (GraphPad Prism Version 5.0).

Results

Peg-IFN α promotes HSPC cell division

In order to characterize the effects of IFN α on human HSPCs *in vivo*, we collected serial samples of bone marrow-derived HSPCs from PV and ET patients being treated with either Peg-IFN α or hydroxyurea. A total of 88 bone marrow samples were obtained from 36 patients randomly assigned to treatment with either hydroxyurea (n=11) or Peg-IFN α (n=26). One patient was converted from Peg-IFN α to hydroxyurea treatment and was therefore counted in both groups. Of the 36 patients, 10 had ET, 25 had PV, and one had MPN not otherwise specified. Bone marrow biopsies were serially obtained from each patient before treatment and during treatment, with a median follow up time of 6 months (range 1-24 months). Each treatment sample was compared to a matched pre-treatment (baseline) sample from the same individual to minimize the effect of patient-specific confounders such as genetic background. Patient characteristics are summarized in **Table 1** and further detailed in **Supplemental Table 1**. For some patients in the hydroxyurea treatment group a pretreatment sample was not available, so samples were compared to a prior hydroxyurea treatment sample from the same individual.

To better understand the effects of IFN α on HSPCs, we confirmed that patients in the Peg-IFN α treatment arm had elevated IFN α levels in serum as determined by ELISA. Three "treatment" samples from Peg-IFN α treated patients who did not have high IFN α levels were excluded from further analysis (**Supplemental Figure 1**). A delay between drug administration and bone marrow aspiration may have explained the low IFN α levels in these samples.

In order to assess the effects of Peg-IFN α on HSPC proliferation, we measured the cell cycle profile of CD34⁺CD45⁺ progenitor cells using Hoechst/Pyronin Y staining (**Figure 1a**). Some of the paired patient samples had insufficient cells for analysis or failed internal controls, leaving a total of 13 paired Peg-IFN α -treated samples and 6 paired hydroxyurea-treated samples for further analysis. Of these, most patients on hydroxyurea treatment demonstrated a significant decrease in the proportion of cells in G2M/S phases of the cell cycle, consistent with its role as a DNA replication inhibitor (**Figure 1b**). In contrast, the majority of patients being treated with Peg-IFN α showed an *increase* in the proportion of cycling HSPCs (**Figure 1b**). Approximately 95% of PV patients and 50% of ET patients carry an activating mutation of *JAK2*^{V617F} in the malignant clone (17), as did most patients enrolled in this study (**Table 1**). We noted that the increase in HSPC cycling seen with Peg-IFN α occurred independently of the starting or ending *JAK2* mutant clonal burden in the samples (*Supplementary Figure 2*), and was particularly noted in one patient (see P24 on heatmap) with no detectable *JAK2* mutation. Thus, the results outlined here can be interpreted to reflect at least the activity of WT clones. When data were analyzed in aggregate, the Peg-IFN α -treated group had a statistically significant ~2-fold increase in the proportion in cell cycle compared to a ~2-fold decrease in the hydroxyurea-treated group (**Figure 1c**). These findings suggest that Peg-IFN α has a positive effect on cell cycle activity of HSPCs, distinct from the suppressive effects of hydroxyurea.

To further assess the effects of Peg-IFN α on cycling of CD34⁺ progenitors, we conducted targeted transcriptional profiling using the Fluidigm microfluidics platform. For these analyses, we also included two paired samples from two untreated controls. As expected, the RNA expression level of the interferon-regulated gene *OAS1* (18, 19) was increased in the cohort of Peg-IFN α treated patients, confirming active IFN α signaling in the treated patients (data not shown). In agreement with the Hoechst/Pyronin Y staining results, RNA expression levels of cell cycle-regulated genes, *CDK6*, *CCNB1*, and *CCNE1* were decreased in patients treated with hydroxyurea but increased in patients treated with Peg-IFN α (**Figure 1d-f**). While individual variation in dose, time to follow up, and comorbid conditions likely contributed to significant variability in the results, the real-time data suggest that the effects of hydroxyurea and Peg-IFN α on HSPC cell division are divergent. While hydroxyurea suppresses cell cycle, in most patients Peg-IFN α promotes proliferation of hematopoietic progenitor cells *in vivo*.

Peg-IFN α promotes HSPC differentiation, not self-renewal

We next assessed total HSPC number by flow cytometry to determine if the balance between self-renewal and differentiation was maintained during Peg-IFN α treatment. There was no significant increase in either the percent or absolute number of CD34⁺CD45⁺CD38⁻ HSPCs in the bone marrow of Peg-IFN α or hydroxyurea treated patients, indicating that neither treatment enhanced HSPC numbers (**Figure 2a-b**). These results indicate that IFN α -induced HSPC proliferation does not result in an increase in phenotypically defined HSPCs.

We then measured progenitor cell differentiation by colony formation assays and transcriptional analysis. First, mononuclear cells were cultured in methylcellulose media with cytokines to allow for the growth of myeloid progenitors. Some samples were not

completed due to contamination of cultures or insufficient cells for analysis, leaving a total of 16 paired IFN α -treated samples and 9 hydroxyurea-treated samples. Of these, the majority of patients treated with Peg-IFN α (12 of 16) showed an increase in the total number of colonies formed (**Figure 2c**), whereas most patients treated with hydroxyurea (6 of 9) had a decrease in colony number (**Figure 2d**). In aggregate there was a statistically significant difference in the log2 fold change in colony number after treatment when comparing the Peg-IFN α versus hydroxyurea treatment groups (**Figure 2e**). Increased colony formation after Peg-IFN α was most notable among PV patients, with little change in colony formation among ET patients. If we included only PV patients treated less than 12 months and for whom the post-treatment sample was collected within one week of drug administration, then the correlation between Peg-IFN α and increased colony formation was 100% (**Figure 2c**). Increased colony formation in response to Peg-IFN α was seen in erythroid, granulocyte, macrophage-specific and multipotent GEMM colonies, whereas hydroxyurea suppressed granulocyte and erythroid colonies specifically (**Figure 2f**). These findings suggest that, in contrast to hydroxyurea, Peg-IFN α promotes differentiation of early progenitors towards multiple cell types.

We also assessed the RNA expression levels of genes required for stem cell self-renewal, *MECOM* (*MDS1-EV11*) and *MPL* (20-22). Notably, both self-renewal genes were down-regulated in CD34+ cells after Peg-IFN α treatment (**Figure 3a-b**), whereas they were unchanged or increased in samples treated with hydroxyurea. These findings are consistent with an IFN α -dependent relaxation of quiescence as noted in prior murine studies (23).

Hydroxyurea and IFN α mediate opposing effects on senescence of HSPCs

In order to determine whether Peg-IFN α induces apoptosis of human HSPCs *in vivo*, we conducted transcriptional analysis of a panel of genes associated with apoptosis. However, we saw no consistent increase in the RNA expression levels of these genes in CD34+ progenitor cells of either the hydroxyurea or Peg-IFN α treated patients (**Figure 3c**). Thus, our RNA expression data are not sufficient to conclude that IFN α induces apoptosis in human HSPCs.

Next, we profiled the expression of genes whose expression correlates with aging, as sustained inflammation and proliferation, such as might occur due to prolonged IFN α exposure, may lead to cellular senescence or aging of HSCs and the molecular profile of older stem cells is linked with decreased stem cell function (24). While these genes tended to be upregulated in HSPCs of patients treated with hydroxyurea, they were consistently unchanged or decreased in HSPCs in the Peg-IFN α treatment group (**Figure 3d**). Thus, hydroxyurea and Peg-IFN α exert opposite effects on the group of senescence-associated genes in HPSCs, again supporting a divergent mechanism of action of these two agents. These findings suggest that senescence is not a major route for HSPC suppression in patients treated with Peg-IFN α .

Discussion

Interferon therapy has been used for the treatment of numerous diseases, including hematologic diseases of stem cell origin such as PV, ET, and CML. Understanding the

effects IFN α has on human HSPCs may lead to improved methods to predict which patients will respond best to Peg-IFN α treatment. In this study, we report the first analysis of the longitudinal molecular response of human hematopoietic progenitors to IFN α in an *in vivo* setting. Our work shows that IFN α activates quiescent progenitors to divide. Moreover, IFN α -induced hematopoietic progenitor proliferation is associated with no change in the number of CD45+ CD34+ CD38– HSPCs, but an increase in colony forming ability in methylcellulose, reflective of myeloid progenitors. These findings lead us to conclude that Peg-IFN α increases differentiation rather than self-renewal of HSPCs.

Our findings validate the relevance of several recent murine studies reporting that IFN α can promote HSC cell division. Essers et al. used rigorous means to show that murine stem cells are induced to divide upon IFN α stimulation (25), and Pietras et al. demonstrated that division occurs through temporary relaxation of quiescence-enforcing machinery in the cells (23). Similarly, we recently showed that IFN γ can promote HSC division and differentiation in mice without altering the overall HSC number (26). We now provide direct evidence that these effects of IFN α occur in human HSPCs.

Although an earlier study showed increased apoptosis in human HSPCs incubated with IFN α *in vitro* (5), our results support an increase in cell division and differentiation without evidence of increased apoptosis *in vivo*. A similar conflict between *in vitro* and *in vivo* studies has been noted previously and was addressed in a recent murine study in which IFN α -treated HSPCs were found to be more susceptible to the effects of secondary stress, including the stress of *ex vivo* culture (23). Indeed, removing HSCs from their *in vivo* niche was sufficient to induce apoptosis in HSPCs previously exposed to IFN α (23, 27). Thus, lack of increased apoptosis in our IFN α -treated samples may be a reflection of their ability to regain homeostasis in the *in vivo* setting. As a caveat though, our study was beset by a number of limitations, specifically differences in the interval of treatment, underlying conditions, treatment dosage, and a small sample size. Thus, we cannot rule out the possibility that apoptosis may have occurred transiently or via posttranscriptionally controlled mechanisms. Similarly, while Peg-IFN α and hydroxyurea treatment appear to have opposing effects on human HSPC senescence, we cannot rule out the aging process as a potential mechanism of remodeling of the hematopoietic compartment at this time.

While as many as 95% of PV patients and 50% of ET patients carry activating mutations of *JAK2* in their diseased HSC clone at the time of diagnosis, the *JAK2* mutant allele burden is often much higher in peripheral cells as compared to HSPCs. Indeed, the overall *JAK2* mutational burden within the CD34+ compartment of our samples was low (<15%). Therefore, we surmise that the differences noted, including increased cell cycle activity and colony formation, are likely representative of the activity of normal wild type (WT) HSPCs. Our findings suggest that increased proliferation and differentiation of WT HSPCs could contribute to the return of polyclonal hematopoiesis in PV and ET patients treated with IFN α . Increasing the division rate of HSCs could also lead to elimination of hyperproliferative PV and ET clones through the effects of secondary p53-mediated or replicative stress (23, 28, 29). Thus, while our study was not designed to address whether IFN α preferentially induces WT HSCs or suppresses *JAK2* mutant clones, the results reported here are highly consistent with both sequelae, as has been seen in mouse models of

JAK2^{V617F}-associated PV(30)(31). Future work using single cell profiling of WT and *JAK2*^{V617F} HSCs will be needed to address these questions.

In conclusion, this study represents the first direct demonstration that IFN α treatment promotes cell cycle activity in HSPCs *in vivo*. This is the basis — until now only theoretical — for use of Peg-IFN α -induced HSC proliferation to increase chemosensitivity in patients with hematologic malignancies (4). This work provides a foundation to improve rational use of IFN α in a number of stem cell-based disorders.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Friedman R. Clinical uses of interferons. *Br J Clin Pharmacol*. Feb 1; 2008 65(2):158–62. [PubMed: 18070219]
2. Kiladjian JJ, Cassinat B, Turlure P, Cambier N, Roussel M, Bellucci S, et al. High molecular response rate of polycythemia vera patients treated with pegylated interferon alpha-2a. *Blood*. 2006; 108:2037–40. [PubMed: 16709929]
3. Hasselbalch HC. A new era for IFN-alpha in the treatment of Philadelphia-negative chronic myeloproliferative neoplasms. *Expert Rev Hematol*. Dec; 2011 4(6):637–55. [PubMed: 22077528]
4. Preudhomme C, Guilhot J, Nicolini FE, Guerci-Bresler A, Rigal-Huguet F, Maloisel F, et al. Imatinib plus peginterferon alfa-2a in chronic myeloid leukemia. *N Engl J Med*. Dec 23; 2010 363(26):2511–21. [PubMed: 21175313]
5. Lu M, Zhang W, Li Y, Berenzon D, Wang X, Wang J, et al. Interferon-alpha targets JAK2V617F-positive hematopoietic progenitor cells and acts through the p38 MAPK pathway. *Exp Hematol*. 2010; 38:472–80. [PubMed: 20303384]
6. Raefsky EL, Platanius LC, Zoumbos NC, Young NS. Studies of interferon as a regulator of hematopoietic cell proliferation. *J Immunol*. 1985; 135:2507–12. [PubMed: 2411798]
7. Chawla-Sarkar M, Lindner DJ, Liu YF, Williams BR, Sen GC, Silverman RH, et al. Apoptosis and interferons: role of interferon-stimulated genes as mediators of apoptosis. *Apoptosis*. 2003; 8:237–49. [PubMed: 12766484]
8. Essers MAG, Offner S, Blanco-Bose WE, Waibler Z, Kalinke U, Duchosal MA, et al. IFNalpha activates dormant haematopoietic stem cells *in vivo*. *Nature*. Apr 16; 2009 458(7240):904–8. [PubMed: 19212321]
9. Baldrige MT, King KY, Boles NC, Weksberg DC, Goodell MA. Quiescent haematopoietic stem cells are activated by IFN- γ in response to chronic infection. *Nature*. Jun 10; 2010 465(7299):793–7. [PubMed: 20535209]

10. King KY, Baldrige MT, Weksberg DC, Chambers SM, Lukov GL, Wu S, et al. Irgm1 protects hematopoietic stem cells by negative regulation of IFN signaling. *Blood*. Aug 11; 2011 118(6): 1525–33. [PubMed: 21633090]
11. Pietras EM, Lakshminarasimhan R, Techner JM, Fong S, Flach J, Binnewies M, et al. Re-entry into quiescence protects hematopoietic stem cells from the killing effect of chronic exposure to type I interferons. *J Exp Med*. 2014; 211:245–62. [PubMed: 24493802]
12. Prchal JT. Polycythemia vera and other primary polycythemia. *Curr Opin Hematol*. Mar 1; 2005 12(2):112–6. [PubMed: 15725900]
13. James C, Ugo V, Le Couédic J, Staerk J, Delhommeau F, Lacout C, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*. Apr 28; 2005 434(7037):1144–8. [PubMed: 15793561]
14. Glimm H, Oh IH, Eaves CJ. Human hematopoietic stem cells stimulated to proliferate in vitro lose engraftment potential during their S/G(2)/M transit and do not reenter G(0). *Blood*. Dec 15; 2000 96(13):4185–93. [PubMed: 11110690]
15. Gaikwad A, Nussenzweig R, Liu E, Gottshalk S, Chang K, Prchal JT. In vitro expansion of erythroid progenitors from polycythemia vera patients leads to decrease in JAK2 V617F allele. *Exp Hematol*. 2007; 35:587–95. [PubMed: 17379069]
16. Nussenzweig RH, Swierczek SI, Jelinek J, Gaikwad A, Liu E, Verstovsek S, et al. Polycythemia vera is not initiated by JAK2V617F mutation. *Exp Hematol*. 2007; 35:32–8. [PubMed: 17198871]
17. James C, Ugo V, Le Couédic JP, Staerk J, Delhommeau F, Lacout C, et al. A unique clonal JAK2 mutation leading to constitutive signalling causes polycythaemia vera. *Nature*. 2005; 434:1144–8. [PubMed: 15793561]
18. Rutherford MN, Hannigan GE, Williams BRCP. Interferon-induced binding of nuclear factors to promoter elements of the 2-5A synthetase gene. *EMBO J*. 1988; 7:751–9. [PubMed: 2456211]
19. Wathlet M, Moutschen S, Cravador A, DeWit L, Defilippi P, Huez G, et al. Full-length sequence and expression of the 42 kDa 2-5A synthetase induced by human interferon. *FEBS Lett*. 1986; 196:113–20. [PubMed: 3753689]
20. Abkowitz JL, Chen JCP. Studies of c-Mpl function distinguish the replication of hematopoietic stem cells from the expansion of differentiating clones. *Blood*. 2007; 109:5186–90. [PubMed: 17347409]
21. Zhang Y, Stehling-Sun S, Lezon-Geyda K, Juneja SC, Coillard L, Chatterjee G, et al. PR-domain-containing Mds1-Evi1 is critical for long-term hematopoietic stem cell function. *Blood*. 2011; 118:3853–61. [PubMed: 21666053]
22. Kimura S, Roberts AW, Metcalf D, Alexander WSCP. Hematopoietic stem cell deficiencies in mice lacking c-Mpl, the receptor for thrombopoietin. *Proc Natl Acad Sci U S A*. 1998; 95:1195–200. [PubMed: 9448308]
23. Pietras EM, Lakshminarasimhan R, Techner JM, Fong S, Flach J, Binnewies M, et al. Re-entry into quiescence protects hematopoietic stem cells from the killing effect of chronic exposure to type I interferons. *J Exp Med*. Feb 10; 2014 211(2):245–62. [PubMed: 24493802]
24. Chambers SM, Shaw CA, Gatz C, Fisk CJ, Donehower L, Goodell MA. Aging hematopoietic stem cells decline in function and exhibit epigenetic dysregulation. *PLoS Biol*. Aug 1.2007 5(8):e201. [PubMed: 17676974]
25. Essers MA, Offner S, Blanco-Bose WE, Waibler Z, Kalinke U, Duchosal MA, et al. IFN α activates dormant haematopoietic stem cells in vivo. *Nature*. 2009; 458:904–8. [PubMed: 19212321]
26. Matatall KA, Shen CC, Challen GA, King KY. Type II Interferon Promotes Differentiation of Myeloid-Biased Hematopoietic Stem Cells. *Stem Cells*. 2014 in press.
27. Mirantes C, Passegue E, Pietras EM. Pro-inflammatory cytokines: Emerging players regulating HSC function in normal and diseased hematopoiesis. *Experimental Cell Research*. Aug 19.2014
28. Beerman I, Seita J, Inlay MA, Weissman IL, Rossi DJ. Quiescent hematopoietic stem cells accumulate DNA damage during aging that is repaired upon entry into cell cycle. *Cell Stem Cell*. Jul 3; 2014 15(1):37–50. [PubMed: 24813857]

29. Dykstra B, Olthof S, Schreuder J, Ritsema M, de Haan G. Clonal analysis reveals multiple functional defects of aged murine hematopoietic stem cells. *J Exp Med.* Dec 19; 2011 208(13): 2691–703. [PubMed: 22110168]
30. Mullally A, Brueedigam C, Poveromo L, Heidel FH, Purdon A, Vu T, et al. Depletion of Jak2V617F myeloproliferative neoplasm-propagating stem cells by interferon- α in a murine model of polycythemia vera. *Blood.* May 2; 2013 121(18):3692–702. [PubMed: 23487027]
31. Hasan S, Lacout C, Marty C, Cuingnet M, Solary E, Vainchenker W, et al. JAK2V617F expression in mice amplifies early hematopoietic cells and gives them a competitive advantage that is hampered by IFN α . *Blood.* Aug 22; 2013 122(8):1464–77. [PubMed: 23863895]

Figure 1. IFNα induces hematopoietic progenitor proliferation

(a-c) The cell cycle profile of CD34+ CD45+ hematopoietic progenitor cells was determined by Hoechst/Pyronin Y staining. Shown is (a) a representative flow plot of Hoechst/Pyronin Y staining and (b) a heatmap of the change in percent of cells in S/G2/M phases of the cell cycle between pre and post-treatment samples for Peg-IFNα or hydroxyurea treated patients. (c) Aggregate log2 fold change in % of cells in G0, G1 and S/G2/M phases. Peg-IFNα (n=13), hydroxyurea (n=6) (d-f) Real-time PCR for the RNA expression levels of cell cycle regulated genes was conducted in CD34-enriched cells from Peg-IFNα, hydroxyurea or untreated patients. Shown are results for (d) *CDK6*, (e) *CCNB1*, and (f) *CCNE1*, which are all upregulated during cell division. Peg-IFNα (n=10), hydroxyurea (n=8), untreated (n=2). All data are shown as the log2 fold change of the treated sample compared to the same patient's pre-treatment sample. * p<0.05 by Student's t-test.

Figure 2. IFN α stimulates differentiation without increasing hematopoietic progenitor number (a+b) The number of CD34+ CD45+ CD38– HSPCs was determined by flow cytometric analysis of whole bone marrow from both hydroxyurea and Peg-IFN α treated patients. Peg-IFN α (n=10), hydroxyurea (n=6). HSPC numbers are shown as both (a) percent of the total live whole bone marrow cells and (b) the absolute number of HSPCs per mL of bone marrow. (c+d) Colony formation was determined from 10⁵ mononuclear cells after 14 days in 2mL methylcellulose media with cytokines to support the growth of myeloid progenitor cells. Shown are paired patient samples for patients treated with Peg-IFN α (c) and for patients on hydroxyurea (d). ET patients are indicated by the green circles; all other patients had PV. Blue circle indicates follow up interval > 12 months; purple circle indicates patient had only received one dose of Peg-IFN α . Yellow square indicates untreated patients started on hydroxyurea; other patients in this cohort were already on hydroxyurea at the time of study enrollment. (e) Aggregate log₂-fold change in colony number. Peg-IFN α (n=16). hydroxyurea (n=9). (f) The breakdown of change in colony formation by colony types is shown here. * p<0.05 by Student's t-test.

Figure 3. IFNα reduces self-renewal but does not induce apoptosis or senescence-associated pathways
 (a-b) Real-time PCR for the RNA expression levels of HSC quiescence genes in CD34-enriched cells from Peg-IFNα, hydroxyurea or untreated patients. Peg-IFNα (n=10), hydroxyurea (n=8), untreated (n=2). (a) *MECOM* (*MDS1-EV11*) expression (b) *MPL* expression. (c) Real-time PCR for the RNA expression levels of apoptosis-associated genes (*APAF1*, *CASP3*, *CASP8*, *CASP9*, *FAS*, *BAD*, *BAX*, *BID*, *MCL1*, *BCL2*) in CD34-enriched cells from Peg-IFNα, hydroxyurea or untreated patients. Peg-IFNα (n=10), hydroxyurea (n=8), untreated (n=2). (d) Real-time PCR for the RNA expression levels of senescence-associated genes (*SELP*, *ICAM1*, *CLU*, *TLR4*, *CTSB*, *PTGS2*, *APP*, *DNAJA2*) in CD34-enriched cells from Peg-IFNα, hydroxyurea or untreated patients. Peg-IFNα (n=24), hydroxyurea (n=11). All data are shown as the log₂ fold change of the treated sample compared to the same patient's pre-treatment sample. * p<0.05; **p<0.01 by Student's t-test.

Table 1

Patient Characteristics	Hydroxyurea (n=11)	PEG-IFN α (n=26) *
Polycythemia vera	8 (73%)	18 (69%)
Essential thrombocythemia	3 (27%)	7 (27%)
Median age	69	61
Male gender	8	13
Median time between samples in months (range)	6 (1-24)	6 (1-24)
Number with <i>JAK2</i> ^{V617F} mutation [†]	5 of 7 (71%)	21 of 23 (91%)
Pretreatment <i>JAK2</i> ^{V617F} allele burden in PV pts: granulocytes (median, range)	53.4% (0-93.3%) n=8	48.4% (2-95.2%) n=16
Pretreatment <i>JAK2</i> ^{V617F} allele burden in PV pts: CD34+ cells (median, range)	11.2% (0-75.6%) n=5	30.7% (1-84.4%) n=15
Pretreatment <i>JAK2</i> ^{V617F} allele burden in ET pts: granulocytes (median, range)	0.8% (0-13.3%) n=3	0% (0-17.3%) n=7
Pretreatment <i>JAK2</i> ^{V617F} allele burden in ET pts: CD34+ cells (median, range)	5.7% (0-11.3%) n=2	1% (0-13.3%) n=7

* One patient had a diagnosis of MPN-NOS.

[†] Not determined for all patients.