Fanca$^{-/-}$ hematopoietic stem cells demonstrate a mobilization defect which can be overcome by administration of the Rac inhibitor NSC23766

Michael D. Milsom,[1] Andrew W. Lee,[1,2] Yi Zheng,[1] and Jose A. Cancelas[1,3]

[1]Division of Experimental Hematology, Cincinnati Children's Hospital Research Foundation, Cincinnati; [2]College of Medicine, University of Cincinnati, Cincinnati, and [3]Hoxworth Blood Center, University of Cincinnati, USA

ABSTRACT

Fanconi anemia is a severe bone marrow failure syndrome resulting from inactivating mutations of Fanconi anemia pathway genes. Gene and cell therapy trials using hematopoietic stem cells and progenitors have been hampered by poor mobilization of HSC to peripheral blood in response to G-CSF. Using a murine model of Fanconi anemia (Fanca$^{-/-}$ mice), we found that the Fanca deficiency was associated with a profound defect in hematopoietic stem cells and progenitors mobilization in response to G-CSF in absence of bone marrow failure, which correlates with the findings of clinical trials in Fanconi anemia patients. This mobilization defect was overcome by co-administration of the Rac inhibitor NSC23766, suggesting that Rac signaling is implicated in the retention of Fanca$^{-/-}$ hematopoietic stem cells and progenitors in the bone marrow. In view of these data, we propose that targeting Rac signaling may enhance G-CSF-induced HSC mobilization in Fanconi anemia.

Key words: Fanconi anemia, mobilization, Rho GTPase, NSC23766, hematopoietic stem cells.

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Introduction

Fanconi anemia (FA) is an inherited disorder characterized by early-onset progressive bone marrow (BM) failure, congenital abnormalities and predisposition towards cancer.[1] On a molecular basis, FA is diagnosed based upon sensitivity to DNA crosslinking agents such as mitomycin C or diepoxybutane.[2] FA patients can be assigned to one of at least 13 complementation groups with 13 corresponding genes having been identified and cloned to date.[3] These proteins are thought to interact in a linear pathway which mediates cellular responses to DNA damage and cellular stress.[4] FA-A is the predominant complementation group and comprises more than 60% of all diagnosed FA patients in North America and Western Europe. FA-A patients develop a 90% cumulative risk of BM failure by 40 years of age[5] and therapeutic regimens have therefore focused upon alleviating this aspect of the disease.[6] In this regard, the only curative therapy currently available is allogeneic stem cell transplantation from a non-affected donor. Successful outcomes of allogeneic transplants implicate that their BM failure is due to HSC impairment and not the hematopoietic microenvironment. Unfortunately, the availability of unaffected sibling donors is low for the majority of patients and the disease-free survival rate for transplant using a matched unrelated donor is not optimal, ranging from 15 to 67%.[7,8] Since many children with FA are diagnosed prior to the onset of severe panycytopenia,[9] a possible novel experimental therapy could use autologous hematopoietic stem cells (HSC) prior to BM failure, for corrective molecular intervention. For instance, these HSC could then be used as a target for retroviral mediated gene replacement therapy, which has been effective in the treatment of other monogenic disorders of the hematopoietic system.[10] Studies which examine the feasibility of collecting hematopoietic stem cells and progenitors (HSC/P) have shown that stem cell mobilization using granulocyte colony-stimulating factor (G-CSF) is not robust in mice,[11] or in FA patients which may require prolonged periods of daily apheresis procedures to obtain clinically relevant numbers of HSC.[12,13] In addition, since the expression of CD34 may not represent an adequate marker of HSC function, the

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Correspondence: Jose A. Cancelas, Division of Experimental Hematology, Cincinnati Children's Hospital Med. Center and Research Division, Hoxworth Blood Center, University of Cincinnati Medical Center, Cincinnati, OH, USA. E-mail: jose.cancelas@chmcc.org

long-term engraftment capability of these HSC has been questioned.[14] Therefore, an alternate approach must be found to enhance collection of FA HSC.

The Rho family of guanosine triphosphatases (GTPases) represents a group of signal transduction proteins which are essential for the regulation of numerous hematologic cellular processes by integrating multiple cell surface receptor signaling pathways into the activation/repression of various effector molecules.[15] Within the Rac subfamily of Rho GTPases, Rac1 and Rac2 have been extensively characterized with regards to their role in regulating the biological properties of HSC including BM retention, migration, homing, proliferation and apoptosis.[16,17] One particularly important observation is that the co-ordinate knockout of both Rac1 and Rac2 results in profound mobilization of HSC from the marrow into the periphery resulting in defective hematopoiesis.[16] This effect can be reversibly reproduced using the small molecule Rac inhibitor NSC23766.[16] Hence, NSC23766 constitutes a novel pharmacologic agent with which to enhance current HSC mobilization protocols.

In this study we utilize the murine Fanca$^{-/-}$ knockout model[18] to investigate whether there is a mobilization defect associated with the disruption of the Fanconi pathway and to determine whether any such defect can be overcome by modulating the mobilization protocol.

Design and Methods

See *The Online Supplementary Appendix*.

Results and Discussion

Fanca$^{-/-}$ mice have a similar HSC content to wild-type littermates based upon immuno-phenotypic markers

In order to characterize the HSC immunophenotype of *Fanca$^{-/-}$* mice, BM cells were harvested from the femora of individual WT and *Fanca$^{-/-}$* mice and stained with antibodies directed against hematopoietic lineage specific markers (CD3, CD4, CD8, B220, Mac-1, Gr-1 and Ter119), c-Kit and Sca-1. As previously reported in a different murine *Fanca$^{-/-}$* model,[19] the frequency and BM content of lineage negative, Sca-1^{+}, c-Kit^{+} (LSK) cells was similar in *Fanca$^{-/-}$* compared with WT mice, as evaluated by flow cytometry (Figure 1A and B). In addition, the level of cell surface expression of several relevant adhesion molecules (CD49d/integrin α_4 chain, CD49e/integrin α_5 chain, CD62L/L-selectin and CXCR4) was not significantly different between WT and *Fanca$^{-/-}$* derived BM (Figure 1C). These data indicate that the frequency of immuno-phenotypically identified BM HSC/P is not impaired in *Fanca$^{-/-}$* mice, and that *Fanca$^{-/-}$* HSC/P express a normal complement of the adhesion receptors involved in HSC engraftment and retention.

Fanca$^{-/-}$ mice have a similar HSC content to wild-type littermates based upon in vivo *repopulation studies*

We next performed functional assays to evaluate the quantity and functions of *Fanca$^{-/-}$* versus WT HSC/P. The homing capacity of *Fanca$^{-/-}$* BM progenitors was first evaluated by measuring the percentage of injected CFU which are able to successfully home to the locale of the BM or spleen of lethally irradiated recipient mice (Figures 2A and B). In accordance with the similar expression profile of cell surface adhesion molecules within the BM LSK fraction, CFU derived from *Fanca$^{-/-}$* and WT donors had comparable homing efficiencies to both the BM and the spleen at 16 h post-transplant.

In order to more definitively assess stem cell function in the context of the *Fanca$^{-/-}$* model, we next carried out a competitive repopulation assay to determine the relative frequency of HSC in *Fanca$^{-/-}$* and WT BM (Figure 2C). At four weeks post-transplant, we observed ~37% lower frequency of competitive repopulating units (CRU) from *Fanca$^{-/-}$* donors compared to WT donors ($p<0.05$). However, at eight weeks *(Online Supplementary Figure 1)* and five months post-transplant (Figure 2C) there was no discernable difference in the frequency of CRU derived from *Fanca$^{-/-}$* or WT donors. Thus, although there is no defect in the homing of *Fanca$^{-/-}$* progenitors to hematopoietic organs, there is a marked

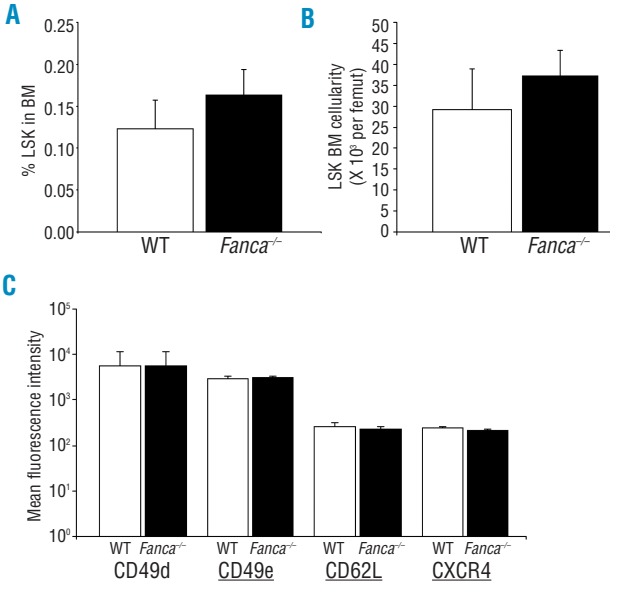

Figure 1. Immunophenotypic characterization of *Fanca$^{-/-}$* versus WT bone marrow (BM) cells. (A) Percentage of LSK cells in the BM. Femora were isolated from either WT or *Fanca$^{-/-}$* mice and were subsequently stained with fluorescent conjugated antibodies directed against Sca-1, c-Kit, and a panel of lineage specific markers, as described in the Methods section. Flow analysis was used to evaluate the percentage of LSK cells in the BM. n=4 per group, in each of two independent experiments. (B) Total number of LSK cells per femur. The absolute number of BM cells per femur was calculated as described in the Methods section. The total femur cellularity was multiplied by the frequency of LSK cells in BM in order to calculate the absolute number of LSK cells per femur. n=4 per group, in each of two independent experiments. (C) Relative expression of cell adhesion molecules in BM LSK population. BM from A was additionally stained with fluorescent antibodies directed against either CD49d, CD49e, CD62L or CXCR4, as described in the Methods section. The relative expression of each of these adhesion molecules within the LSK population was assessed by determining the mean fluorescent intensity of staining by flow analysis. n=4 per group, in each of two independent experiments. □ WTBM; ■ *Fanca$^{-/-}$* BM.

defect in the number of *Fanca*$^{-/-}$ BM derived short-term (ST) repopulating cells. Nonetheless, in *Fanca*$^{-/-}$ mice the frequency of more primitive BM cells which contribute to long-term (LT) reconstitution is normal.

Fanca$^{-/-}$ HSC demonstrate a profound defect in G-CSF-mediated mobilization

We next sought to evaluate the propensity of *Fanca*$^{-/-}$ HSC/P to be mobilized into the periphery following treatment with G-CSF. In accordance with the data generated in human clinical trials, G-CSF treatment led to a more than 70% reduction in CFU per mL of peripheral blood compared with WT mice (p<0.01, Figure 3A). The decreased progenitor mobilization of *Fanca*$^{-/-}$ mice was also associated with a decreased mobilization of HSC into the blood. By analyzing the competitive repopulating activity of peripheral blood following G-CSF treatment, we observed a more than 90% lower mobilization of *Fanca*$^{-/-}$ HSC compared to WT (p<0.01), as evaluated at four months post-transplant (Figure 3B). BM harvested from these primary recipient mice was subsequently transplanted into secondary recipients in order to evaluate the mobilization of self-renewing LT-HSC. Secondary recipients of *Fanca*$^{-/-}$ G-CSF mobilized peripheral blood demonstrated a more than 45% lower

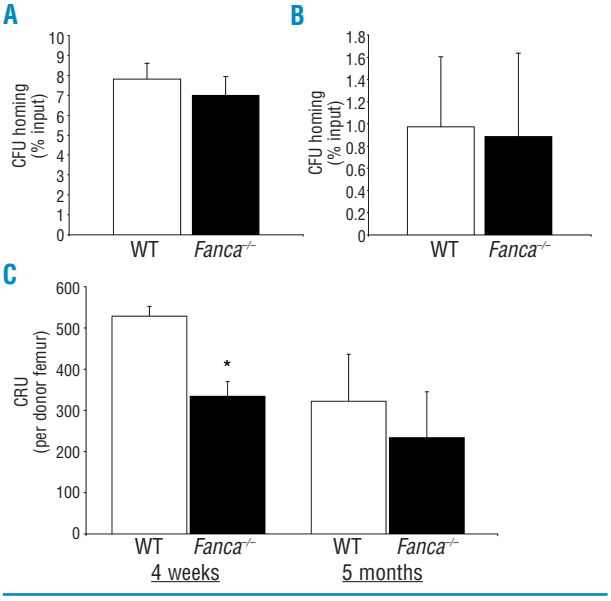

Figure 2. Functional characterization of *Fanca*$^{-/-}$ versus WT BM cells. (A) CFU homing to the bone marrow (BM). Lethally irradiated mice were injected with 1×10^7 *Fanca*$^{-/-}$ or WT BM, and 16 h later, BM was isolated from the femora and tibiae of recipient mice and plated out in methylcellulose. Homing efficiency is expressed as the percentage of CFU which were recovered from the BM relative to the number of CFU which were injected into the recipient mice. n=5 per group, in each of two independent experiments (B) CFU homing to the spleen. At the time of sacrifice, splenocytes were harvested from the recipient mice described in A. Splenocytes were plated out in methycellulose, and, as for BM, the homing efficiency was calculated as the percentage of CFU recovered from the spleen relative to the number of CFU injected into the recipient mice. n=5 per group, in each of two independent experiments (C) Competitive repopulation activity of *Fanca*$^{-/-}$ versus WT BM. BM was harvested from the femora of age matched WT or Fanca$^{-/-}$ mice (both CD45.2$^+$). Lethally irradiated recipient mice (CD45.1$^+$) were co-injected with 3×10^6 competitor BM cells (CD45.1$^+$) and either 1/10 femur equivalent of Fanca$^{-/-}$ BM or 1/10 femur equivalent of WT BM. At both four weeks and five months post-transplant, recipient mice were bled and the percentage of CD45.2$^+$ cells in the peripheral blood was determined by flow analysis after staining with fluorescent labeled antibodies. The number of CRU was calculated as described in the Methods section. Data is from one representative experiment of two independent experiments performed. *p<0.05, n=5-6 per group. □ WT BM; ■ *Fanca*$^{-/-}$ BM.

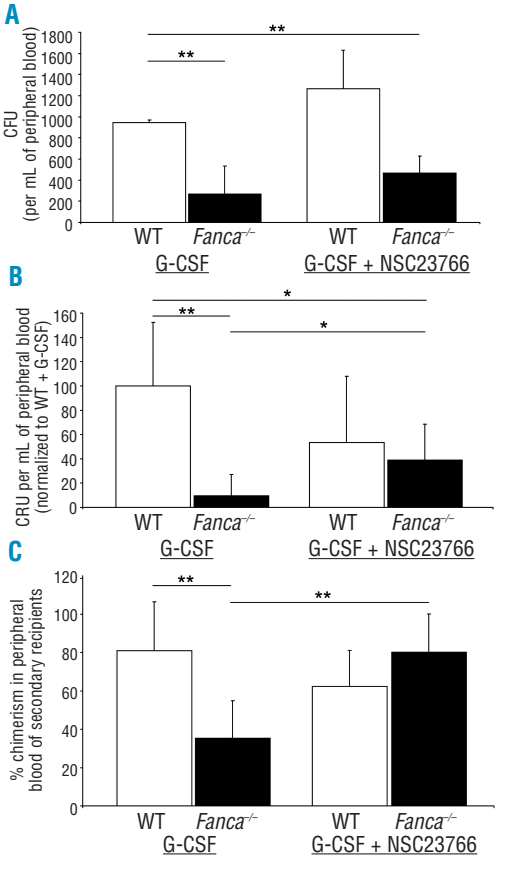

Figure 3. Relative mobilization of *Fanca*$^{-/-}$ versus WT HSC/P. (A) Mobilization of CFU via treatment with G-CSF alone, or G-CSF and NSC23766. *Fanca*$^{-/-}$ or WT mice were treated with either G-CSF alone, or G-CSF and NSC23766 as described in the Methods section. Peripheral blood was isolated from treated mice via cardiac puncture. 100 μL of peripheral blood was subject to red cell lysis and was then plated out in methycellulose. CFU were scored seven days later. **p<0.01, n=3 per group, from two independent experiments. (B) Mobilization of CRU via treatment with G-CSF alone, or G-CSF and NSC23766. *Fanca*$^{-/-}$ or WT mice were treated with G-CSF alone or G-CSF and NSC23766 as described above. 400 μL of peripheral blood was subject to red cell lysis and was then co-injected into lethally irradiated CD45.1$^+$ recipient mice along with 5×10^5 competitor (CD45.1$^+$) bone marrow (BM) cells. At four months post-transplant, recipient mice were bled and the percentage chimerism of CD45.2$^+$ cells was determined by staining with fluorescent antibodies prior to flow cytometry as described in the Methods section. The CRU per ml of peripheral blood was determined as described in the Methods section (normalized to the frequency of CRU in the WT group mobilized with G-CSF alone). *p<0.05, **p<0.01, n=6-8 mice in each experimental group from two independent experiments. (C) Secondary transplant of BM from competitive transplant recipients. BM was isolated from the primary competitive transplant recipients described in B, and 1×10^7 BM cells were injected i.v. into lethally irradiated secondary recipients (CD45.1$^+$). At six months post-transplant, peripheral blood was harvested from recipient mice and the percentage of donor chimerism (CD45.2$^+$) was determined as above. **p<0.01, n=5-7 mice in each experimental group from two independent experiments. □ WT mobilized peripheral blood; ■ *Fanca*$^{-/-}$ mobilized peripheral blood.

donor (CD45.2$^+$) chimerism than secondary recipients of WT mobilized peripheral blood (Figure 3C, $p<0.01$). Taken together, these results indicate that *Fanca*$^{-/-}$ HSC/P are not efficiently mobilized in response to administration of G-CSF.

The Fanca$^{-/-}$ *mobilization defect can be overcome at the level of LT-HSC by co-administration of NSC23766*

In an effort to establish whether the *Fanca*$^{-/-}$ mobilization defect could be overcome by inhibition of the Rac GTPase pathway, we administered the small molecule Rac inhibitor NSC23766 to WT and *Fanca*$^{-/-}$ mice. For both WT and *Fanca*$^{-/-}$ mice, there was no further mobilization of CFU into the peripheral blood when the G-CSF treatment was supplemented with NSC23766 in either WT or *Fanca*$^{-/-}$ mice (Figure 3A, p=N.S. for WT G-CSF treated *vs.* WT G-CSF+NSC23766 and for *Fanca*$^{-/-}$ G-CSF treated *vs. Fanca*$^{-/-}$ G-CSF+NSC23766). However, when the frequency of HSC in the periphery was enumerated by competitive repopulation assay (Figure 3B), the combination of G-CSF and NSC23766 resulted in >4-fold increase in the number of mobilized *Fanca*$^{-/-}$ CRU over G-CSF treatment alone ($p<0.05$). In this mouse strain, we observed no additional effect upon G-CSF mobilization of WT CRU when NSC23766 was included in the treatment regimen (p=NS). Altogether, these data indicate that Rac inhibition mediated by NSC23766 co-operates with G-CSF in the mobilization of HSC from *Fanca*$^{-/-}$ BM.

Finally, we performed secondary transplants using BM harvested from the primary competitive transplants described above. There was no difference in donor chimerism (CD45.2$^+$) in secondary recipients of WT peripheral blood which had been mobilized with either G-SCF or G-CSF and NSC23766 (Figure 3C, p=NS). However, the addition of NSC23766 to the G-CSF mobilization protocol resulted in >2-fold increase in Fanca$^{-/-}$ donor (CD45.2$^+$) chimerism in secondary recipients ($p<0.01$). Notably, the peripheral blood product of *Fanca*$^{-/-}$ mobilization with G-CSF and NSC23766 together effected an equivalent reconstitution of secondary recipients, compared to the product of WT mobilization with either G-CSF alone or G-CSF and NSC23766 (p=NS).

Thus, an experimental mobilization protocol consisting of G-CSF and NSC23766 treatment results in enhanced mobilization of *Fanca*$^{-/-}$ ST-HSC and LT-HSC over a conventional G-CSF mobilization protocol. Novel mobilization protocols are required for patients with a poor stem cell reserve. This is particularly evident in cases where cell or gene therapy protocols are intended to be applied. These technologies rely on the use of large numbers of starting stem cells prior to any *ex vivo* manipulation to ensure successful engraftment. The use of autologous HSC from FA patients as a means to correct progressive BM failure via experimental therapeutic intervention requires an effective minimally invasive method to collect such large amounts of cells. Clinical trials have demonstrated the limited effectiveness of G-CSF induced mobilization to effect the release of primitive hematopoietic cells into peripheral blood from the BM niche in the context of early-diagnosis FA patients with no evidence of BM failure.[12,13] Therefore we have tested a novel approach to stem cell mobilization in a murine model of FA.

Initially, we determined that *Fanca*$^{-/-}$ BM is defective in ST-HSC but not LT-HSC compared with BM from WT littermates. Thus, *Fanca*$^{-/-}$ mice demonstrate a discrete stem cell defect with no evidence of BM failure.

We then found that *Fanca*$^{-/-}$ mice displayed a severe mobilization defect in response to G-CSF treatment when compared to WT littermates. Recently, CXCR4-dependent signaling has been found to be normal in HSC derived from *Fanca*$^{-/-}$ mice,[20] suggesting that the major axis of HSC retention in BM is intact and is therefore not responsible for the phenomenon we describe. An alternate explanation for our data could be extrapolated from the observation that mobilization has been previously defined as a sensitive approach to evaluate the total reserve of HSC in the context of an artificially-induced stem cell defect.[21] Thus, Fanca$^{-/-}$ mice may have a subtle HSC defect which cannot be discerned directly by competitive repopulation assay, yet is manifested as a profound mobilization defect. Regardless of the underlying mechanism, the *Fanca*$^{-/-}$ model is analogous to the situation in early diagnosis FA patients who fail to mobilize efficiently but have no evidence of BM failure.[12]

We therefore tested the ability of the combined use of G-CSF and NSC23766 to mobilize Fanca$^{-/-}$ HSC/P. Interestingly, NSC23766 appeared to rescue the mobilization defect shown by *Fanca*$^{-/-}$ mice upon administration of G-CSF, but did not increase the mobilization effect of G-CSF administration in WT mice. This suggests that *Fanca*$^{-/-}$ BM contains a subset of HSC which are unresponsive to G-CSF but can be mobilized upon administration of a Rac activation inhibitor.

Others have previously reported the use of AMD3100, a CXCR4 inhibitor, in order to enhance HSC mobilization in a different murine *Fanca*$^{-/-}$ model. In this model, targeting the *CXCR4/CXCL12* signaling axis successfully rescued a defective mobilization in response to G-CSF.[11] Intervention on Rac activity offers the attractive advantage that it is likely to interfere with not only the *CXCL12/CXCR4* signaling axis but also with the chemoattractant role of SCF and the adhesion function of β1-integrins, which are all located upstream of Rac.

In summary, the studies reported here show that Rac GTPases represent molecular targets for therapeutic manipulation of transplantable stem cells and in combination with G-CSF can rescue the mobilization failure in an animal model of FA. We propose that mobilization protocols which target the inhibition of Rac signaling may be clinically useful in the harvest of HSC/P from patients who have suffered inherited or acquired BM failure.

Authorship and Disclosures

MDM performed research, data analysis and interpretation, and wrote the manuscript. AWL performed research, data analysis and interpretation. YZ provided

study materials and contributed to data analysis and final approval of manuscript. JAC conceived and designed the study, performed data analysis and interpretation, wrote the manuscript and approved it. YZ is the inventor of NSC23766, which is protected by U.S. Patent.

The authors reported no other potential conflicts of interest.

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