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AMD3100 synergizes with G-CSF to mobilize repopulating stem cells in Fanconi anemia knockout mice

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Abstract

Fanconi anemia (FA) is a heterogeneous inherited disorder characterized by a progressive bone marrow (BM) failure and susceptibility to myeloid leukemia. Genetic correction using gene transfer technology is one potential therapy. A major hurdle in applying this technology in FA patients is the inability of granulocyte colony-stimulating factor (G-CSF) to mobilize sufficient numbers of hematopoietic stem (HSC)/progenitor cells (HPC) from the BM to the PB. Whether the low number of $CD34^+$ cells is a result of BM hypoplasia or an inability of G-CSF to adequately mobilize FA HSC/HPC remains incompletely understood. Here we use competitive repopulation of lethally irradiated primary and secondary recipients to show that in two murine models of FA, AMD3100 synergizes with G-CSF resulting in a mobilization of HSC, whereas G-CSF alone fails to mobilize stem cells even in the absence of hypoplasia.

Keywords

Stem cells; Fanconi anemia; Stem cell mobilization; AMD3100; Stem cell transplantation

Introduction

Fanconi anemia (FA) is a heterogeneous genetic disorder characterized by a progressive bone marrow (BM) aplasia, chromosomal instability and the acquisition of malignancies. The progressive BM failure and the late developing myeloid malignancies account for 90% of the mortality in FA [1]. Aside from cord blood transplantation [2], the only cure for the hematopoietic manifestations of FA is an HLA identical allogeneic BM transplantation from a family member, a therapy available to only about 30% of patients [3]. Thirteen FA complementation types (FANCA, FANCB, FANCC, FANCD1, FANCD2, FANCE,

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FANCF, FANCG, FANCI, FANCJ, FANCL, FANCM and FANCN) have been identified and the cDNAs of twelve FA genes have been sequenced [4–28]. The identification of these genes raises the potential of using gene transfer technology to express the functional cDNA in autologous stem cells. The BM hypoplasia commonly observed in FA patients [1] and the reduced repopulating ability of stem cells in mice containing a disruption of the murine homologue of an FA gene [29,30] led to the hypothesis that autologous, genetically-corrected stem cells would have an engraftment and proliferation advantage.

Since many of these patients are small children, mobilization of autologous stem cells from BM into peripheral blood (PB) for collection would be ideal. Granulocyte colony-stimulating factor (G-CSF) is an FDA approved therapy utilized for mobilization of hematopoietic stem (HSC)/progenitor cells (HPC) for autologous transplantation in a variety of clinical settings [31]. However, patients with FA show a markedly decreased response to G-CSF [32]. The inability of G-CSF to adequately mobilize HSC/HPC in FA patients has prompted the search for alternative methods.

The interaction between BM stroma and HSC via SDF-1/CXCL12 and CXCR4, respectively, plays an important role in HSC chemotaxis and retention within the marrow [33]. AMD3100, a bicyclam derivative, is a competitive antagonist of this interaction and thus serves as a potential agent for HSC/HPC mobilization [34]. AMD3100 works synergistically with G-CSF to induce mobilization of the following: wild type murine HSC, NOD/SCID repopulating cells from normal human donors and HPC from *Fancc* −/− mice [34]. Further, the combination of G-CSF and AMD3100 has been administered to patients with multiple myeloma and has been shown to be an efficacious and safe regimen for stem cell mobilization [35]. We examined the effect of AMD3100 and G-CSF on HSC mobilization in FA types A and C murine models. We establish that the combination of AMD3100 and G-CSF works synergistically to mobilize HSC with long-term BM repopulating ability and self-renewal capacity in two murine models of FA.

Materials and Methods

Experimental animals

Fanca −/− mice and *Fancc* −/− mice on a C57BL/6J background were used as detailed in previous studies [36,37]. Congenic wildtype C57BL/6J (WT) mice and wildtype B6.SJL-Ptrca Pep/BoyJ (BoyJ) mice were purchased from Jackson ImmunoResearch Laboratories. The Institutional Animal Care and Use Committee approved all studies.

Drug administration and isolation of low-density mononuclear cells

G-CSF (Amgen Corp.) was administered at a dose of 3 μg subcutaneous (*s.c.*) in 0.1 ml phospho-buffered saline/0.1% bovine serum albumin (PBS/0.1% BSA) every 12 hours for four consecutive days [34]. Control animals received a similar volume of PBS/0.1% BSA for four consecutive days. AMD3100 (provided by Dr. Gary Bridger, AnorMed, Inc., Langley, British Columbia) was administered at a dose of 5 mg/kg *s.c.* 14 hours following the last dose of G-CSF and one hour prior to the collection of PB. Low-density mononuclear cells (LDMC) were isolated from the PB using Ficoll Hystopaque-1119 (Sigma), washed in PBS/0.1% BSA and resuspended in Iscove's Modified Dulbecco's Medium (IMDM) with 10% fetal bovine serum (FBS).

Hematopoietic progenitor cell assays

LDMC from each genotype/treatment group were cultured in 1.04% methylcellulose in IMDM supplemented with 30% FBS, 200 U/ml penicillin/streptomycin and 80 μM 2-mercaptoethanol. Assays were performed in triplicate and were supplemented with the

following cytokines: 50 ng/ml murine SCF, 10 ng/ml murine GM-CSF, 100 U/ml murine IL-3 (Peprotech) and 2U/ml human recombinant EPO (Amgen Corp.). Cells were cultured for 7 days at 37°C, 5% CO_2 and then scored for the presence of CFU-GM and BFU-E/CFU-GEMM hematopoietic progenitor colonies.

Competitive repopulation assays

Competitive repopulation experiments were conducted as previously described in primary and secondary recipients [36,38]. Briefly, LDMC from 1 ml (cohort 1) or 2 ml (cohort 2) of the PB of donor WT, *Fanca* −/− or *Fancc* −/− mice treated with G-CSF, AMD3100 or both were transplanted along with 7.5×10^5 BoyJ competitor BM LDMC into WT (8–12 week old) lethally irradiated recipients (1100 rads). These two independent experiments with 3–5 recipients/genotype/treatment group were analyzed. Chimerism was measured monthly as described previously [36]. Briefly, PB cells were collected and stained with FITC-conjugated anti-CD45.2 antibodies (BD Biosciences) and the percentage of positive cells was determined by flow cytometry. Four months after transplant, 2×10^6 LDMC were collected from the BM of primary recipients and transplanted into lethally irradiated secondary recipients (1100 rads) to test self-renewal capacity as described previously [34]. Multi-lineage analysis was performed on the BM LDMC from primary recipients using FITC-conjugated anti-CD45.2 and PE-conjugated anti-CD3, anti-Mac1 or APC-conjugated anti-Gr1 and anti-B220 antibodies (BD Biosciences). The percentage of cells double positive for CD45.2 and the specific lineage marker was determined using flow cytometry.

Statistical analysis

Results are expressed as mean ± 1 standard error of the mean (SEM). Statistical differences between groups were determined using analysis of variance with Bonferroni correction. Repopulating units (RU) were calculated using RU= (competitor number × %$CD45.2^+$)/(100−%$CD45.2^+$) [39].

Results

In previous studies, increased mobilization of hematopoietic progenitors in *Fancc* −/− mice was assessed; however, the broad applicability of this approach to other FA genotypes was not determined. Given potential subtle differences in the phenotypes of FA genotypes in man and various FA knockout mice, we validated and extended our studies in *Fanca* −/− mice, the murine homologue of the most prevalent FA complementation group in man. Analogous to previous studies in WT and *Fancc* −/− mice, administration of G-CSF and AMD3100 resulted in at least an additive mobilization of lineage restricted and multipotent hematopoietic progenitors as compared to use of either compound alone (Figure 1). Furthermore, an increase in the mobilization of lineage restricted and multipotent progenitors was observed in *Fanca* −/− and *Fancc* −/− mice.

Previous studies in *Fancc* −/− mice examined the mobilization of progenitors but not repopulating HSC [34]. Given that mobilization of HSC is required for effective gene therapy in FA, we next evaluated the ability of G-CSF and AMD3100 alone or in combination to mobilize repopulating stem cells in WT, *Fanca* −/− and *Fancc* −/− mice using competitive repopulation. A schematic of the experimental design is shown in Figure 2. Lethally irradiated recipients received mobilized peripheral blood cells from donor mice ($CD45.2^+$) along with Boy J ($CD45.1^+$) competitor BM cells. CD45.1 and CD45.2 chimerism was analyzed monthly in order to determine the competitive repopulating ability of mobilized test cells. The repopulating ability of fresh BM from the three respective genotypes was also calculated. The results of these data are shown in Figure 3 and Table I. As expected, the repopulating ability of the BM from *Fanca* −/− and *Fancc* −/− mice was

only 35–40% of syngeneic WT controls (Table I). Strikingly, there was a much greater defect in the repopulating activity of *Fanca* −/− and *Fancc* −/− PB cells following mobilization with G-CSF alone (15–30% of WT controls) analogous to the paucity of $CD34^+$ phenotypic cells mobilized using G-CSF only in FA patients [32] and the reduced numbers of lineage restricted and multipotent progenitors. Though stem cells mobilized with AMD3100 alone resulted in low chimerism in all experimental groups (Figure 3), addition of G-CSF to AMD3100 resulted in a greater than additive increase in the chimerism and repopulating ability of *Fanca* −/− and *Fancc* −/− HSC (Figure 3, Table I).

To verify that repopulating HSC with self-renewal capacity were mobilized to PB, primary recipients were sacrificed four months following transplantation and BM LDMC from these recipients were transplanted into lethally irradiated secondary recipients. No significant change in chimerism occurred following transplantation into secondary recipients (Figure 4). To determine whether the donor cells contributed to multiple blood lineages, multi-color flow cytometry was performed. Analysis of $CD45.2^+$ PB cells with anti-B220, CD3, Gr-1 and Mac-1 antibodies demonstrated the presence of multi-lineage lymphoid and myeloid derived $CD45.2^+$ donor cells. An analysis from a representative recipient is shown in Figure 5 and the data is summarized in Table II. Collectively, the results support the hypothesis that AMD3100 plus G-CSF resulted in mobilization of long-term, multi-lineage, self-renewing HSC.

Discussion

FA remains a complex genetic disorder with significant morbidity and mortality [1]. Patients frequently develop BM failure in the second decade of life [1]. HLA-matched BM or cord blood transplantation remains the only curative option; however, only approximately one-third of patients are able to find an HLA-matched donor [3]. Therefore, there is a great need to develop other treatments. Genetic correction and transplantation of autologous HSC is a potential therapy; however, mobilization of adequate HSC in FA patients is a key prerequisite for non-invasive collection. Previous trials in FA patients using extended G-CSF administration mobilized a limited number of $CD34^+$ cells [32]. It was unclear whether the relative inability of G-CSF to mobilize $CD34^+$ cells in FA patients is a consequence of BM hypoplasia or a defect in stem cell/stromal cell interactions. This question is difficult to address in human patients; however, the availability of syngeneic FA knockout mice provided the opportunity to address this issue in a preclinical setting.

Here, we quantitatively examined the repopulating ability of the BM and mobilized PB HSC in syngeneic FA and WT mice. As expected from previous studies [29,37,40], both *Fanca* −/− and *Fancc* −/− mice had a significant decrease in bone marrow repopulating ability as compared to the repopulating ability of WT mice. However, in response to GCSF alone, this decrease in repopulating ability was accompanied by an additional decrease in the proportion of repopulating units mobilized from the bone marrow to the peripheral blood. Our results demonstrate that mobilization with both AMD3100 and G-CSF results in a synergistic increase in the mobilization of long-term, multi-potential, self-renewing HSC. Ongoing studies in our laboratories are addressing the potential of these cells as targets for gene transfer in FA murine models and in human NOD-SCID repopulating cells.

Data from this study demonstrate that mobilization of PB repopulating stem cells with G-CSF is clearly defective in FA knockout mice. One possibility is that FA deficient hematopoietic stem cells have an inability to respond to a number of chemokines that are important for stem cell mobilization. However, in preliminary studies, we have found that the responsiveness of myeloid progenitors to a number of cytokines and chemokines (including stem cell factor, G-CSF and stromal cell-derived factor-1) have failed to discern

such a phenotype (unpublished data, 2007). Alternatively, it has recently been shown that hematopoietic progenitor and stem cell mobilization is dependent on a complex model involving both protease dependent and independent mechanisms [41–44]. Given our observations in these studies and the fidelity with which the murine models recapitulate the human mobilization defects, the FA murine models may be useful in future studies to discern the basic mechanisms underlying this clinical phenotype.

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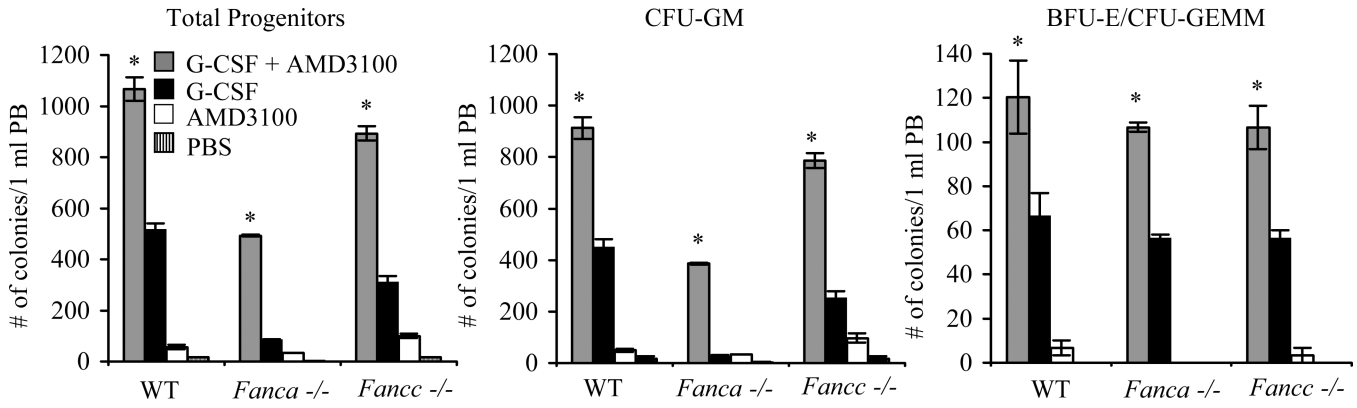

Figure 1. Mobilization of HPC is enhanced when both G-CSF and AMD3100 are used

The mean total number of HPC/ml PB ± SEM of the indicated lineages and genotypes are shown n=3/genotype, *p<0.05.

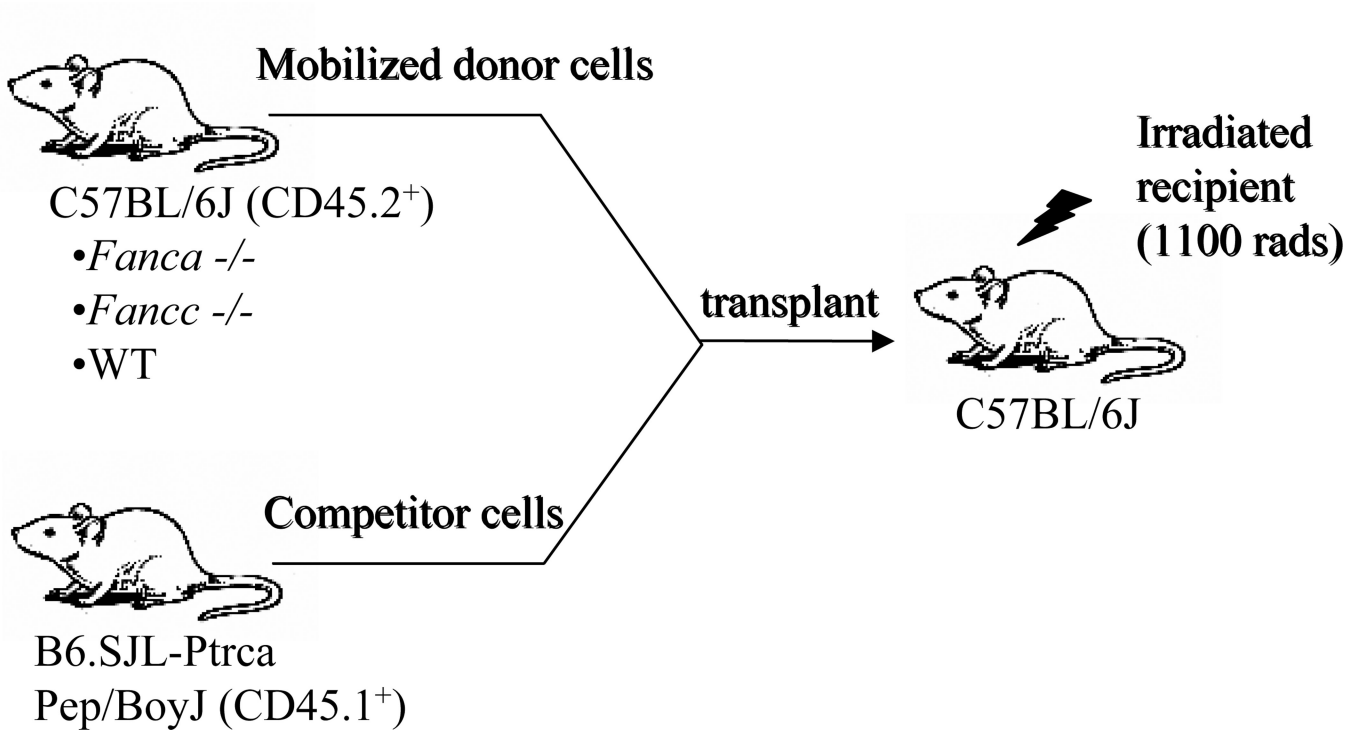

Figure 2. Competitive repopulation

Mobilized donor cells (CD45.2$^+$) are collected from the PB following mobilization and transplanted along with BoyJ competitor BM cells (CD45.1$^+$) into lethally irradiated recipients. The percentage of donor-derived cells in the PB was measured monthly.

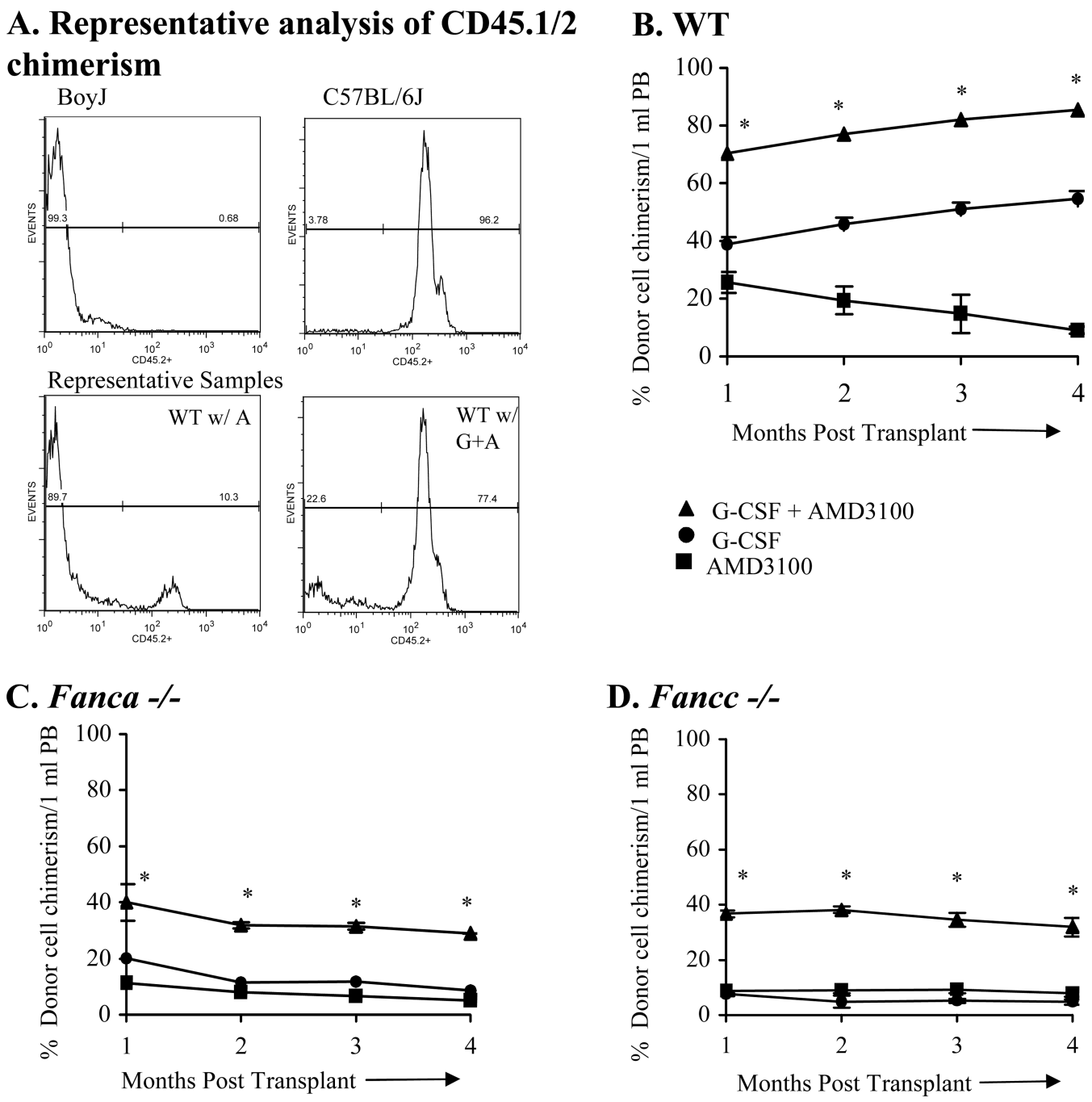

Figure 3. Mobilization of HSC is enhanced when G-CSF and AMD3100 are used in combination

Flow cytometry analysis was used to determine percentage of donor-derived chimerism in the PB of recipient mice (A). Percent donor cell chimerism of each genotype and mobilization group are indicated (B–D). Results are shown as mean ± SEM. n=5; *p<0.05.

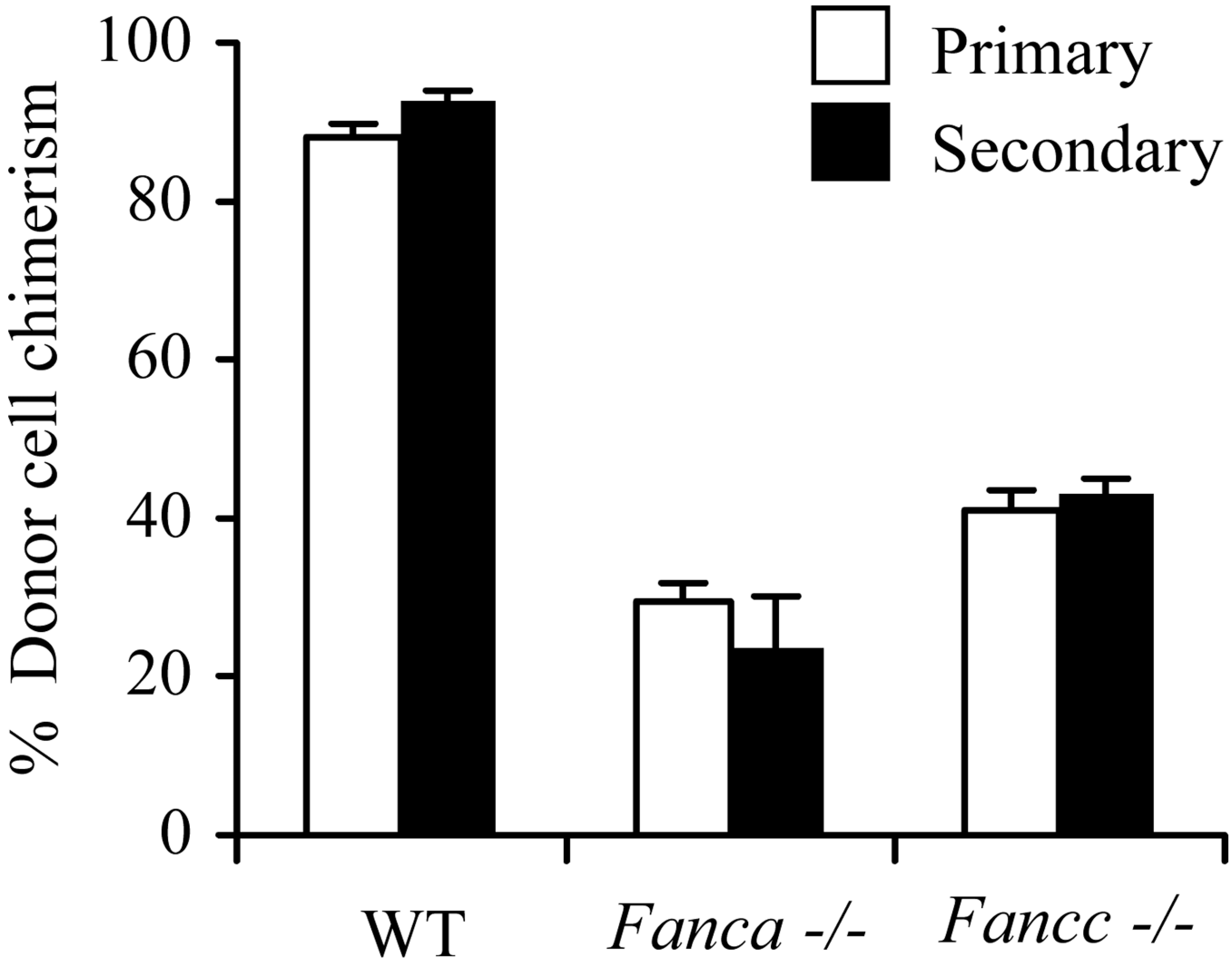

Figure 4. Secondary transplants showed no significant change in chimerism, implying the mobilized cells had self-renewal capacity

Results depict chimerism 4 months post transplantation for primary recipients (white bars) receiving G-CSF plus AMD3100 mobilized cells and chimerism 4 months post transplantation for secondary recipients (black bars). n=3 for primary recipients, n=6 for secondary recipients, $p>0.05$.

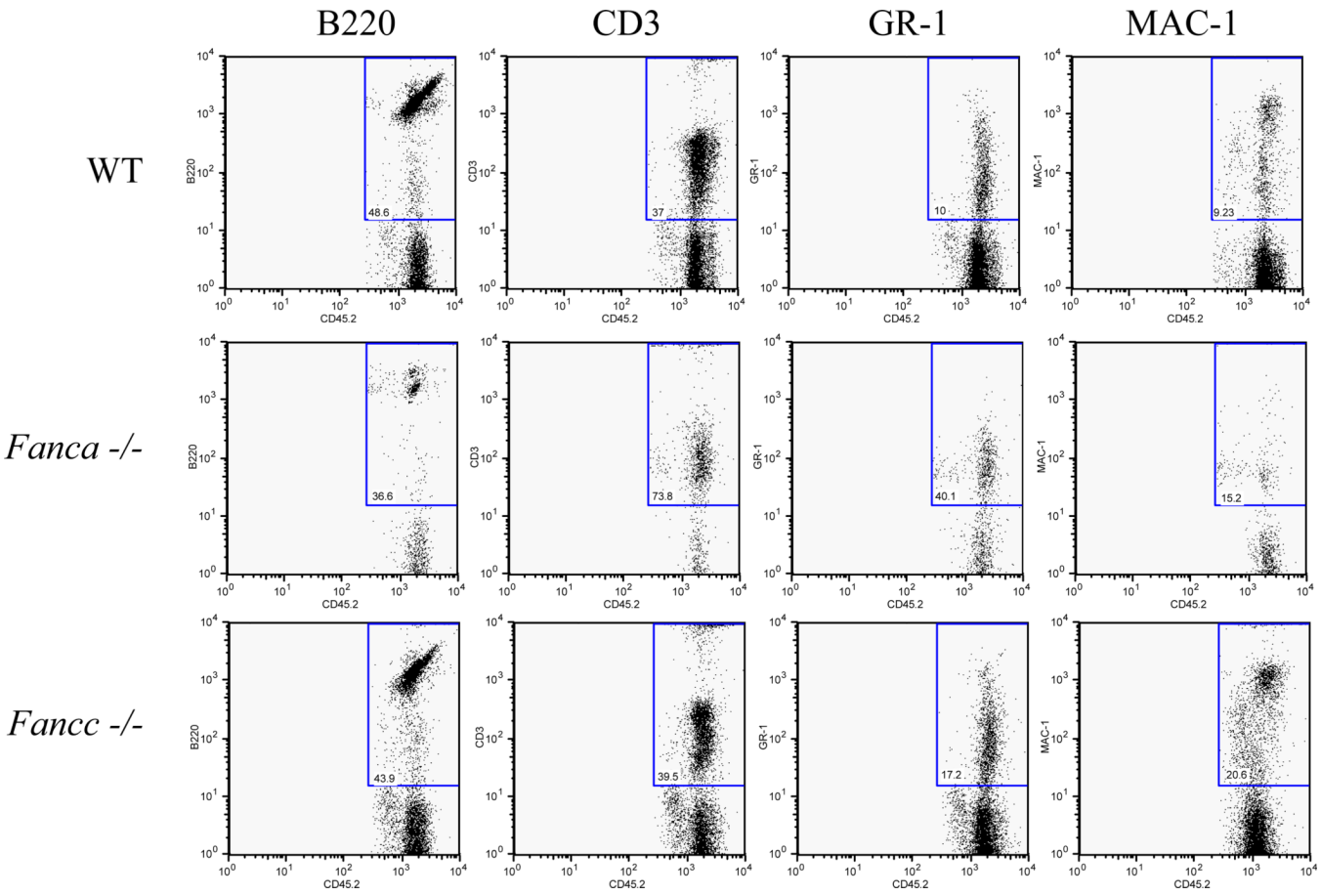

Figure 5. G-CSF plus AMD3100 mobilized HSC are capable of sustaining multi-lineage reconstitution

CD45.2$^+$ PB cells from primary recipients receiving either WT, *Fanca* −/− or *Fancc* −/− HSC mobilized with G-CSF and AMD3100 were analyzed for CD3, B220, Gr-1 and Mac-1 expression. Results depicted above are representative samples gated on CD45.2$^+$ in order to show the percentage of CD45.2$^+$ PB cells expressing the respective antigen.

Table I

Repopulating units of BM and mobilized cells from WT, *Fanca* −/− and *Fancc* −/− mice.

	BM	RU/1 ml PB		
	RU/Femur	G-CSF	AMD3100	G-CSF & AMD3100
WT	72.30±0.17	4.12±0.89	1.90±0.62	10.66±0.58
Fanca **−/−**	25.29±0.06	0.67±0.03	0.51±0.13	2.57±0.12
Fancc **−/−**	26.02±0.10	1.23±0.57	0.47±0.12	3.21±0.32

Table II

Multi-lineage differentiation of AMD3100 + G-CSF mobilized PB cells in recipient mice.

	CD3	B-220	GR-1	MAC-1
WT	21 ± 6	24 ± 7	31 ± 7	24 ± 7
Fanca **−/−**	26 ± 4	22 ± 6	21 ± 6	30 ± 5
Fancc **−/−**	29 ± 4	34 ± 3	16 ± 5	30 ± 6

$CD45.2^+$ PB cells from primary recipients receiving either WT, *Fanca* −/− or *Fancc* −/− mobilized HSC were analyzed for CD3, B220, Gr-1 and Mac-1 expression 4 months post transplantation. Results depicted are mean percentages of chimeric $CD45.2^+$ PB cells expressing the respective antigen ± SEM. n=5.