Impaired functionality and homing of Fancg-deficient hematopoietic stem cells

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Fanconi anemia (FA) is a human rare genetic disorder characterized by congenital defects, bone marrow (BM) failure and predisposition to leukemia. The progressive aplastic anemia suggests a defect in the ability of hematopoietic stem cells (HSC) to sustain hematopoiesis. We have examined the role of the nuclear FA core complex gene Fancg in the functionality of HSC. In Fancg−/− mice, we observed a decay of long-term HSC and multipotent progenitors that account for the reduction in the LSK compartment containing primitive hematopoietic cells. Fancg−/− lymphoid and myeloid progenitor cells were also affected, and myeloid progenitors show compromised in vitro functionality. HSC from Fancg−/− mice failed to engraft and to reconstitute at short and long term the hematopoiesis in a competitive transplantation assay. Fancg−/− LSK cells showed a loss of quiescence, an impaired migration in vitro in response to the chemokine CXCL12 and a defective homing to the BM after transplantation. Finally, the expression of several key genes involved in self-renewal, quiescence and migration of HSC was dysregulated in Fancg-deficient LSK subset. Collectively, our data reveal that Fancg should play a role in the regulation of physiological functions of HSC.

INTRODUCTION

Fanconi anemia (FA) is a recessive disease characterized by congenital defects, progressive bone marrow (BM) failure and predisposition to cancer, including acute myeloid leukemia and squamous cell carcinoma. Progressive occurrence of anemia constitutes the main cause of mortality in FA patients. Fifteen genes (FANCA, FANCB, FANCC, FANCD1/BRCA2, FANCD2, FANCE, FANCF, FANCX/RCC9, FANCI, FANCJ/BRIP1/BACH1, FANCN/PALB2, FANCO/Rad51c and FANCN/SLX4) have been identified whose deficiency appears to contribute to the FA phenotype (1). FA cells show hypersensitivity to DNA crosslinker agents and the FA pathway is involved in DNA repair. In response to DNA damage, eight FA proteins form a nuclear core complex responsible for the mono-ubiquitination of FANCD2 and FANCI (the ID complex). Once mono-ubiquitinated, the ID complex is recruited to DNA repair foci, where it colocalizes with DNA repair proteins, such as BRCA1 and Rad51. The loss of any core-complex components results in defective ubiquitination of the ID complex (2). FANCX/RCC9 is a 65 kDa protein.

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with at least seven protein–protein interaction tetratricopeptide repeat motifs (3). This protein is a component of the nuclear FA complex. Fancc−/−, Fanca−/−, Fancc−/− and Fancd2−/− mice show similar phenotypes including cellular hypersensitivity to mitomycin C (MMC), spontaneous chromosome breakage and reduced fertility (4,5). However, the phenotype appears to be less severe than in humans as the mice show a normal life span and no occurrence of anemia.

Homeostasis of the hematopoietic tissue is maintained throughout the life by a rare population of hematopoietic stem cells (HSC) (6). In the BM, the niche regulates their self-renewal and differentiation to give rise to the different blood cell lineages. The genomic integrity of stem cells is fundamental to maintain the stem cell pool and tissue function (7), and some DNA repair abnormalities are linked to stem cell dysfunction (8). As a DNA repair syndrome, the FA pathway was recently involved in neural progenitors and stem cells failure (9). However, increased production of reactive oxygen species (ROS), overactivation of stress-activated protein kinase and enhanced sensitivity to cytokines like tumor necrosis factor-alpha and interferon-gamma were also reported to play a role in FA pathology (10–14). The progressive BM failure in FA patients suggests a defect in the proliferation and the differentiation of primitive hematopoietic cells. Although anemia is not observed spontaneously in FA knockout models, BM failure was induced in Fancc−/− mice after treatment with the DNA damaging agent MMC (15). In addition, HSC from Fancc−/− and Fancd2−/− mice and from mice with a hypomorphic mutation in Fancd1 displayed altered self-renewal potential and reconstitution ability using a competitive repopulation assay into mouse myeloablated recipients (16–18). Recently, double-mutant Fancc−/−; Fanca−/− mice were reported to develop more severe hematologic phenotypes, including BM failure and hematologic neoplasms found in FA patients (19). Moreover, HSC from Fancc−/− mice were not able to reconstitute hematopoiesis after ex vivo culture expansion (20), and hypoxia/reoxygenation exposure was shown to induce premature senescence in FA hematopoietic cells (21).

In this paper, we investigated the influence of deletion of Fancc on the functionality of HSC and primitive hematopoietic progenitors (HPC). We found that the loss of Fancc resulted in a reduction in HSC and HPC subsets. Fancc-deficient HSC failed to reconstitute at short- and long-term hematopoiesis after transplantation, and notably show defects in quiescence, in migration in vitro in response to chemokine CXCL12 and in BM homing.

RESULTS

Fancc−/− mice display alterations in hematopoietic progenitors/stem cells compartments

Analysis of peripheral blood showed similar platelet counts, white and red blood cell counts, hematocrit, mature T and B lymphocytes and circulating myeloid cells in adult wild-type (WT) and Fancc−/− mice (Table 1 and Supplementary Material, Fig. S1). BM cellularity was also normal in Fancc−/− adult mice (respectively, 43 ± 5 × 10⁶ and 44 ± 4 × 10⁶ cells

<table>
<thead>
<tr>
<th>Lineage</th>
<th>WT (n = 4)</th>
<th>KO (n = 4)</th>
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<tr>
<td>Leukocytes, × 10⁶/ml</td>
<td>12.36 ± 1.48</td>
<td>12.80 ± 0.52</td>
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<tr>
<td>Red blood cells, × 10⁹/ml</td>
<td>10.75 ± 0.19</td>
<td>10.84 ± 0.29</td>
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<tr>
<td>Hematocrit, %</td>
<td>49.85 ± 0.73</td>
<td>50.04 ± 1.49</td>
</tr>
<tr>
<td>Platelets, × 10ⁱ²/ml</td>
<td>772.3 ± 55.23</td>
<td>673 ± 24.64</td>
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Differences are not statistically significant (n = 4).

for WT and Fancc−/− mice cells flushed from two femurs and two tibias per mouse, n = 8). All these data indicate that Fancc deficiency did not induce gross abnormalities of hematopoiesis.

Analysis of the HSC and early progenitor compartments showed a 30% decrease in LSK cells in Fancc−/− BM (Fig. 1A and B). This decrease was mostly accounted for by a 41% decreased number of multipotent progenitors (MPP) (LSK CD34⁺ Flk-2⁻) associated with a normal number of short-term HSC (ST-HSC, LSK CD34⁺ Flk-2⁻) and a 34% decreased number of long-term HSC (LT-HSC, LSK CD34⁻ Flk-2⁻). Fancc-deficient mice had a 2-fold reduced number of common lymphoid progenitors (CLP) (Fig. 2A), and analysis of the committed myeloid progenitor compartments showed only a slight reduction in common myeloid progenitors (CMP) cells and granulocyte/macrophage progenitors (GMP) numbers but a significant 2-fold decreased number of megakaryocyte/erythrocyte progenitors (MEP) in Fancc−/− BM (Fig. 2B).

In vitro clonogenic potential of myeloid committed progenitors (colony-forming cells, CFCs) was also examined. Unfractionated BM cells from Fancc−/− mice displayed a reduced number and a smaller size of colonies compared with WT controls (Fig. 3A and B). The decrease in the colony size suggested a proliferative defect of those progenitors. Multipotent colony-forming unit granulocyte erythrocyte macrophage (CFU-GEMM), bipotential colony-forming unit granulocyte macrophage (CFU-GM) and unipotential colony-forming unit granulocyte (CFU-G) and colony-forming unit macrophage (CFU-M) colonies were reduced showing a global decrease in the capacity of progenitors towards myeloid differentiation (Fig. 3C). A decay of the colony-forming unit erythroid (CFU-E) and burst-forming unit erythroid (BFU-E) colonies was also observed (Fig. 3D). In the same way, methylcellulose cultures performed from sorted Fancc−/− LSK cells produced a lower number of colonies compared with controls, and confirmed that the functional defects concerned early hematopoietic progenitors and affected all myeloid lineages (Fig. 3E and F). Collectively, these data demonstrate that the hematopoietic stem/progenitor cell compartment is altered in the BM of Fancc−/− mice.

Functional long-term defects of HSC in Fancc−/− mice

To assess the functionality of HSC from Fancc−/− mice, we performed competitive stem cell reconstitution experiments using EGFP Fancc−/− and EGFP WT mice in order to discriminate donor cells from competitor and recipient cells. First, total BM cells from either EGFP Fancc−/− or EGFP
Figure 1. Hematopoietic stem/progenitor cell defects in Fancg−/− mice. (A) Phenotypic analysis of the HSC/HPC subsets. Plots from one representative animal from each genotype are shown. HSC/HPC subset, LSK: long-term HSC, LSK CD34+ Flk-2−; short-term HSC LSK CD34+ Flk-2−; MPP: LSK CD34+ Flk-2−. (B) Frequency (%) of long-term HSC (LSK CD34+Flik-2−), short-term HSC (LSK CD34+Flik-2−) and MPP (LSK CD34+Flik-2−) in function of total BM cells. n = 7–8 mice per group.
WT were mixed at a 1:1 ratio with total BM cells from WT mice and injected intravenously into lethally irradiated WT recipients. Flow cytometry analysis of EGFP CD45-positive cells in peripheral blood 4, 8, 16 and 24 weeks after transplantation revealed that EGFP $Fancg^{2/-}$ HSC exhibited a strong defect in their ability to reconstitute at short term and long term the hematopoiesis in irradiated recipients (Fig. 4A and B). The average percentage of repopulation 24 weeks after transplantation was 43 and 10% for WT and $Fancg^{-/-}$ donor cells, respectively. In another set of experiments, competitive stem...
cell reconstitution assays were performed with 2000 LSK cells from either EGFP $Fancg^{2/2}$ or EGFP WT mice mixed with 250,000 BM cells from WT mice. By analysis of peripheral blood, a similar deficit in the ability of LSK $Fancg^{2/2}$ cells to reconstitute hematopoiesis was observed (Fig. 4C). Moreover, this defect was not associated with a block in the differentiation process of a specific lineage, since lymphoid and myeloid lineages were affected in the same manner (Fig. 4D). Furthermore, the frequency of $Fancg^{-/-}$ donor-derived LSK population was low in the BM of the recipients 16 weeks after transplantation (Fig. 4E). These data show an impairment of the regenerative potential of $Fancg^{-/-}$ HSC.

In $Lig4$- and $Fancd1$-deficient mice, an increase in the number of empty niches in the BM due to HSC defect in physiological...
conditions was reported. These niches, being available for transplanted HSC, allowed the engraftment of donor HSC without conditioning of the recipient (18,22). In order to test this hypothesis in Fancg−/− mice, WT EGFP-positive BM cells were transplanted intravenously in unconditioned 4-month-old Fancg−/− and WT recipient mice. No difference of engraftment was observed between Fancg−/− and WT recipient mice 1, 2 and 4 months after transplantation (data not shown), which surmised that niches were occupied by endogenous HSC in the BM of Fancg−/− mice in homeostatic conditions.

Collectively, these data highlight a functional defect of Fancg−/− HSC in regenerative conditions.

Figure 4. Loss of Fancg impairs short- and long-term repopulation potential of HSC. (A) Flow analysis of EGFP chimerism of CD45+ cells in blood of irradiated recipient mice 24 weeks after competitive transplantation with either Fancg−/− EGFP or EGFP WT mixed at a 1:1 ratio with BM cells from WT mice. Representative plots of one recipient animal transplanted with cells from each genotype are shown. (B) Comparison of short- and long-term engraftment of transplanted Fancg−/− EGFP and EGFP WT BM cells in competitive repopulation experiments. Proportion of CD45+ EGFP donor-derived cells in blood of recipient mice (n = 9 except for t = 4 weeks where n = 6, statistically significant difference, P < 0.0007, was found at any time between the two groups). (C) CD45+ EGFP donor-derived cells chimerism in peripheral blood of recipient mice transplanted with 2,000 LSK cells from either EGFP Fancg−/− or EGFP WT mice in competitive repopulation assay (n = 4–7, a statistical significant difference, P < 0.03, was found at any time between the two groups). (D) Proportion of donor-derived lymphocytes T (LT4-8), lymphocytes B (B220) and myeloid (MAC-1, GR-1) of either mutant or WT origin in peripheral blood of recipient mice 24 weeks after transplantation (n = 4). (E) Donor-derived LSK cells in BM from recipient mice 6 months after transplantation of EGFP Fancg−/− LSK (mean frequency is indicated, n = 3).
Impaired function of HSC is frequently associated with the loss of quiescence and excessive proliferation (23,24). To address a potential alteration of the cell-cycle regulation in the stem/progenitor cell compartment, we performed the analysis of the proliferative cell marker ki-67 combined with DNA content in LSK population, allowing to discriminate ki-67-negative quiescent cells from ki-67-positive proliferating cells (Fig. 5A). In the LSK subset, the ratio between quiescent G0 cells and cells that have entered cell cycle in G1 phase was lower for Fancg−/− than for WT, showing a loss of quiescence (Fig. 5B). Thus, Fancg seemed to be involved in the balance between metabolically active and inactive cells of the HSC/HPC pool at the steady state. In addition, the exhaustion of the HSC/HPC pool did not appear to reflect decreased survival as apoptosis of LSK cells was similar in Fancg−/− and WT mice as determined by using fluorescent annexin-V (Fig. 5C).

**Figure 5.** Fancg deficiency impairs quiescence in HSC/HPC compartment. (A) Flow analysis of DNA content and Ki-67 expression in LSK subset from WT and Fancg−/− mice. (B) Proportion of quiescent cells in LSK populations from WT and Fancg−/− mice according to the flow analysis of intracellular ki-67 expression combined with DNA content (n = 5–6 per group). (C) Frequency of apoptotic cells in WT and Fancg−/− LSK cells measured by fluorescent annexin-V assay (n = 3). (D) Proportion of quiescent cells in LSK populations from WT and Fancg−/− mice according to the NAC treatment. Data obtained from the flow analysis of intracellular ki-67 expression combined with DNA content (n = 5–6 per group). (E) Comparison of short-term and long-term engraftment of recipient mice transplanted with either EGFP Fancg−/− or EGFP WT BM cells from control mice and mice treated with NAC in competitive repopulation experiments. A statistically significant difference, *P < 0.0001*, was found at any time between the two groups (n = 6–8 per group).
Anti-oxidant NAC treatment does not restore \( Fancg^{−/−} \) 
HSC potential

Several lines of evidence suggest a role for oxidative stress in the establishment of the FA pathology (10). Moreover, ROS were reported to inhibit the repopulating capacity and to reduce the lifespan of HSC in mice (25). Therefore, a role of ROS in the \( Fancg^{−/−} \) HSC defect was investigated. First, we did not detect any difference in levels of intracellular ROS between LSK cells from \( Fancg^{−/−} \) and WT mice (data not shown). Nevertheless, we examined the effect of dietary supplementation with the anti-oxidant N-acetyl-L-cysteine (NAC) in order to investigate whether HSC defects under physiological conditions might result from chronic exposure of HSC to low increase of endogenous ROS that we could not detect in our assay. NAC was added to drinking water of \( Fancg^{-/-} \) deficient mice from fertilization continuously throughout the life in order to also protect HSC during mouse development. Even if a partial recovery of the quiescent status of LSK was observed in NAC-treated \( Fancg^{−/−} \) mice (Fig. 5D), HSC from NAC-treated and control \( Fancg^{−/−} \) mice continued to show a strong defect in their ability to engraft and repopulate at short- and long-term irradiated recipients fed with NAC (Fig. 5E). Similar defects of engraftment were obtained when adult \( Fancg^{−/−} \) mice were only treated with NAC for 1 month before transplantation (data not shown). Altogether, our data show that intrinsic HSC deficiency in \( Fancg^{−/−} \) mice should not be linked to an exposure to increased intracellular ROS.

Mice lacking \( Fancg \) show a defect in migration in response to CXCL12 and homing to the BM

A deficit of hematopoietic reconstitution at short term and long term could result from impaired migration/homing of HSC to the BM niche after transplantation. Interestingly, we observed that \( Fancg \) deficiency might affect migratory capacity of mouse embryonic fibroblasts (MEF). In a wound-healing assay, wound closure after scratch wounding of a monolayer of MEF was indeed reduced in \( Fancg^{−/−} \) MEF (Supplementary Material, Fig. S2). The proliferation rates of WT and \( Fancg^{−/−} \) MEF were similar at early passages, indicating that the defect of migration of \( Fancg^{−/−} \) MEF cannot be attributed to difference in cell proliferation (Supplementary Material, Table S1). Hence, we investigated the migration capacities of HSC/HPC from \( Fancg^{−/−} \) mice. The chemokine CXCL12 is one of the main chemoattractant factor involved in the homing of HSC to the BM (26). We tested the migration of \( Fancg^{−/−} \) LSK cells in response to CXCL12. LSK cells were placed in the top well of a transwell chamber, and the chemokine-containing medium was placed in the bottom well. Migrating cells were collected from the bottom well and counted (Fig. 6A). A significant decrease in migration to CXCL12 gradient was observed in \( Fancg^{−/−} \) LSK cells compared with the WT. This defect did not appear to be linked to a downregulation of the CXCL12 receptor, i.e. CXCR4, since CXCR4 mRNA was expressed at the same level in WT and \( Fancg^{−/−} \) LSK cells (Fig. 6B). As CXCL12 is a key protein for the first steps of the engraftment after transplantation, \( Fancg^{−/−} \) LSK cells were tested for their capacity to home in the BM \textit{in vivo}. Using a new fibered confocal fluorescence imaging system, we tracked \textit{in vivo} the early steps of recruitment to the BM of either EGFP \( Fancg^{−/−} \) or EGFP WT LSK transplanted intravenously into lethally irradiated mice (27). This imaging system can navigate inside the femoral cavity from the knee to the femoral head of living mice and was used to visualize the hematopoietic reconstitution within the femoral cavity after lethal irradiation. Recruitment of the transplanted LSK first occurs in the femoral head and is continuous during 24 h after the transplantation. By endoscopic imaging of the femur, EGFP-positive LSK cells that migrated into the femur were detected and counted 18 h after transplantation of 1000 LSK cells. We observed that fewer EGFP \( Fancg^{−/−} \) LSK cells successfully homed to the femur when compared with EGFP LSK cells from WT mice (Fig. 6C and D). Hence, deletion of \( Fancg \) in LSK cells resulted in an impairment in their ability to home to the BM microenvironment. Endoscopic imaging of the femur 9 days after transplantation showed the presence of EGFP \( Fancg^{−/−} \) cell clusters (>15 μm), even though fewer cell clusters were observed compared with femurs transplanted with EGFP WT cells (Supplementary Material, Fig. S3). However, these data suggest that \( Fancg^{−/−} \) LSK cells, which succeeded in homing to the BM after transplantation, could proliferate and produce hematopoietic cell clusters in the BM. These data highlight the defective homing of \( Fancg^{−/−} \) LSK cells.

HSC engraftment assays are based on intravenous injection, and require circulation through blood, adhesion and transmigration through BM vasculature (28). We surmised that the injection of HSC directly in the femur cavity should bypass some of these crucial steps of homing. Hence, we investigated whether intrafemoral injection of HSC could rescue the deficit of hematopoietic regeneration at long term. BM cells from either EGFP \( Fancg^{−/−} \) or EGFP WT mice were mixed at a 1:1 ratio with BM cells from WT mice and injected directly in femurs from lethally irradiated WT recipients. Sixteen weeks after transplantation by intrafemoral route, recipient mice displayed a chimerism in peripheral blood ≈10% (Fig. 6E) and lymphoid and myeloid lineages were both severely impaired (Fig. 6G). Likewise, the EGFP chimerism of LSK cells was low in BM of recipients transplanted with EGFP \( Fancg^{−/−} \) BM cells when compared with transplantation with EGFP WT BM cells (respectively, 37 ± 7 and 12 ± 7%; n = 8–9 per group). The transplantation by intrafemoral route did not restore the ability of \( Fancg^{−/−} \) HSC to regenerate hematopoiesis. An excessive apoptotic cell death of isolated \( Fancg^{−/−} \) HSC during the transplantation process might explain the defective repopulation capacity that we observed. However, we detected similar levels of apoptosis in transplanted EGFP \( Fancg^{−/−} \) LSK and EGFP WT LSK cells 20 h after intrafemoral transplantation (Fig. 6F). Hence, these data show that an increased cell death in \( Fancg^{−/−} \) HSC was not responsible for their defective repopulation capacity after transplantation.

Impaired homing/retention to the niche of HSC in several genetic mouse models is sometimes associated to altered frequency of HSC/HPC in peripheral blood. Circulating HSC
Figure 6. Loss of Fancg impairs in vitro migration to CXCL12 and in vivo homing to the BM of LSK cells. (A) In vitro migration of Fancg−/− and WT LSK cells in response to CXCL12 (n = 9). (B) Cxcr4 mRNA expression in Fancg−/− and WT LSK cells. The relative expression is shown normalized to gene expression in control WT LSK. (C) Analysis of the homing of LSK cells to BM obtained by endoscopy of the femur using a confocal fluorescence imaging system. The graph shows the mean number of EGFP-positive LSK cells that were present in the femur 18 h after intravenous transplantation of EGFP Fancg−/− or EGFP WT LSK cells (n = 4 femurs). (D) Representative intrafemoral confocal fluorescence images of EGFP-positive LSK cells from Fancg−/− or WT mice that migrated to the femur after transplantation in irradiated recipient mice. (E) Comparison of short- and long-term engraftment of either EGFP Fancg−/− or EGFP WT LSK cells transplanted in competitive assay via intrafemoral route. Statistically significant difference, P < 0.019, was found at any time between the two groups (n = 10–14 per group). (F) Apoptosis in EGFP-positive LSK cells 20 h after intrafemoral transplantation of BM cells from either EGFP Fancg−/− or EGFP WT mice in femurs from lethally irradiated WT recipients (n = 6). (G) Proportion of donor-derived lymphocytes T (LT4-8), lymphocytes B (B220) and myeloid cells (MAC-1, GR-1) of either mutant or WT origin in peripheral blood of recipient mice 4 months after intrafemoral transplantation (n = 8–10 per group). (H) Graph showing the frequency of LSK cells in the peripheral blood of WT and Fancg−/− mice (n = 8).
are contained within the LSK fraction in the peripheral blood. We detected a decreased number of circulating LSK in the peripheral blood of Fancg−/− mice compared with the WT (Fig. 6H), underlying that steady-state egress of HSC/HPC should also be impaired in Fancg−/− mice.

Collectively, our data demonstrate that Fancg-deficient HSC/HPC show defects in the complex process of homing/migration to the niche in the BM.

**Fancg deficiency deregulates several genes involved in self-renewal, quiescence and migration**

Alteration in HSC capacities due to Fancg−/− deficiency could result from changes in expression levels of key regulators of their physiology. In this regard, recent reports suggest a role of the FA core complex in the regulation of gene transcription (29,30). LSK cells from Fancg−/− and WT mice were sorted and analyzed by real-time quantitative RT–PCR for several genes involved in the physiology of HSC (Fig. 7). No changes were observed for cell-cycle regulators p27, p18 and p19 and transcription factors like c-myc, MEF and Hif1-α. However, the Bmi1 polycomb group gene was downregulated in LSK Fancg−/− mice. This analysis revealed also changes in the Notch signaling pathway. Notch1 was slightly up-regulated, though the Notch target gene Hes1 was markedly downregulated. We also observed in Fancg−/− LSK subset an increase in the cyclin-dependent kinase inhibitor p21^waft/cip1, a gene repressed by HES-1. Since p21^waft/cip1 increase could also result from higher levels of DNA damage (31), we examined in LSK cells from Fancg−/− mice the presence of γH2AX foci which are robust markers of DNA strand breaks. We did not find any increase in DNA damage as similar levels of γH2AX foci were generated in LSK cells from WT and Fancg−/− mice (Supplementary Material, Fig. S4). Real-time quantitative RT–PCR analysis also revealed a significantly decreased expression of N-cadherin, but not of cdc42 and Rac1, molecules that have been shown to be critical for homing of HSC (32–34). EGR1 (early growth-response factor-1) is involved in the regulation of the proliferation and mobilization of HSC (35). In Fancg−/− mice, the zinc-finger transcription factor Egr1 and the cell-cycle regulator p57Kip2 were downregulated in LSK cells and suggested that it could be involved in the decreased pool of quiescent LSK. Taken together, those data show that several key cell cycle, self-renewal and adhesion regulators coordinating the quiescence and the homing of HSC are altered in Fancg−/− HSC/HPC.

**DISCUSSION**

As shown by the significantly decreased ability to engraft lethally irradiated recipient mice, the regenerative potential of Fancg-deficient HSC is severely impaired. The deletion of Fancg reduced the number of HSC/HPC, compromised the quiescent status of HSC/HPC, caused defective migration to chemokine CXCL12 and contributed to defective homing to the BM niche. Those data highlight the role of Fancg in the functionality of HSC.

Although hematopoiesis appeared normal in Fancg−/− mice, the HSC/HPC compartment of the BM displayed several defects. We observed in Fancg−/− mice decreased frequencies of LSK and LSK CD34− Flk-2− HSC subsets. Myeloid progenitors from Fancg−/− mice were affected and show in vitro compromised functionality. A diminished capacity to produce CFU colonies was previously reported for Fancd2−/− mice and mice with a hypomorphic mutation in Fancd1, but not for the mice deficient for the FA core proteins A and C (16–18,36), suggesting a specific role of Fancg in the differentiation of myeloid progenitors among the FA nuclear core proteins. Fancg deficiency resulted also in a reduction in the bipotent MEP. In addition to compromising myeloid progenitors, we found that Fancg−/− BM contained fewer MPP and CLP progenitors, indicating a potential role of Fancg in lymphopoiesis. Likewise, compromised lymphoid-primed MPP or CLP were observed in Fanc−/− and Fanc2−/− mice (16,36).

Consistent with the 34% decreased number of LT-HSC in Fancg−/− mice, we observed a decay of their potential to reconstitute long-term hematopoiesis in competitive transplantation with total BM cells. However, this dramatic decrease in regeneration (~80%) was not simply due to the reduction in phenotypically defined LT-HSC in Fancg−/− mice, but was also associated with a clear reduction in the functionality of LT-HSC. As we found similar numbers of ST-HSC in the BM of WT and Fancg−/− mice, the strong impairment of regeneration at short term is also related to altered functionality of Fancg−/− ST-HSC. Impaired functionality of ST-HSC and LT-HSC was confirmed when LSK cells from WT and
Fancg−/− mice were transplanted in competition with total BM cells. Collectively, our data showed clearly that Fancg-deficient HSC have a cell-autonomous defect in their ability to reconstitute irradiated mice, although it was recently suggested that FA proteins could also be required in the microenvironment of the BM to sustain hematopoiesis (36,37). In several mouse models showing HSC deficiency, a relationship was reported between the impairment of the repopulation capacity after transplantation and a loss of quiescence. Indeed, the primitive quiescent HSC have the most efficient capacity of long-term reconstitution (38). Activated HSC should be responsible for the daily maintenance of the hematopoiesis, i.e. tissue homeostasis. Quiescent subset should constitute a reservoir of stem cells which could be activated after tissue injury in order to recover stem cell pool and hematopoiesis (6). The phenotype of Fancg−/− mice could reflect this dual partitioning of the HSC pool in function of the cell-cycle status. We found that Fancg deficiency resulted in a decreased proportion of quiescent LSK cells showing that Fancg is involved in the balance between metabolically active and inactive cells of the HSC/HPC pool, as previously described in Fancc−/− and recently in Fancd2−/− mice (36,39). On one hand, this lack of dormancy could contribute to the strong deficiency of Fancg−/−/HSC to regenerate hematopoiesis at long term when transplanted in lethal irradiated recipient. On the other hand, homeostasis of hematopoietic tissue could be maintained by activated HSC which should explain that no gross hematopoietic abnormalities were observed in adult Fancg−/− mice. The increased proliferation of the Fancg−/− LSK population could reflect a compensation mechanism to maintain the homeostasis of the hematopoietic tissue as a consequence of the loss of stem cells. However, Fancg−/− mice could show higher susceptibility to tissue injury and aging, because of the decrease in the reservoir of dormant HSC. In agreement with this, Fancg−/− mice, but not WT mice, treated with the DNA cross-linking agent MMC displayed progressive reduction in clonogenic and LTC-IC progenitors, leading to BM failure (15).

The Drosofila hairy and enhancer of split (HES) 1 basic helix-loop-helix protein is a major downstream effector in the canonical Notch pathway, which is surmised to play a role in the self-renewal and maintenance of HSC, and in the control of quiescence (40,41). Interestingly, FA core complex was recently shown to regulate Hes1 transcription and HES1 target genes (29,30). In agreement with this, we also observed in Fancg−/− LSK subset a decrease in Hes1 expression and an increase in a target gene repressed by HES-1, the cyclin-dependent kinase inhibitor p21^waf1/cip1. This upregulation of p21^waf1/cip1 is a hallmark of FA mutant cells (42). Its role in HSC is controversial and a recent work shows that p21^waf1/cip1 seems not to be essential during steady-state hematopoiesis (43). Nevertheless, the increase in p21^waf1/cip1 in the context of telomere dysfunction in Terc−/− HSC, which induces DNA damage signaling, was in part responsible for the impairment of the repopulation capacity of HSC (44).

Oxidative stress, which is a critical determinant of HSC lifespan and self-renewal (24,25), was reported to play potentially a role in the establishment of the FA pathology (10). In line with this, the protein FANCG interacts with the mitochondrial peroxidase peroxiredoxin 3, which is an important cellular antioxidant that regulates physiological levels of H2O2 (45). However, the dietary supplementation with the thiol-containing antioxidant, NAC of Fancg−/− mice did not rescue the HSC defect of engraftment when they were transplanted. Altogether, our data show that intrinsic HSC deficiency in Fancg−/− mice should not be linked to an increase in intracellular ROS, although it was recently described a partial recovery of colony-forming unit spleen after treatment of Fancd2−/− mice with revestralon, an activator molecule of SirT1 deacetylase with antioxidant activity (36).

Transplanted HSC circulate in the blood flow and have to interact with endothelial cells from specific microvasculature structures through sinusoids in order to enter in the BM and to locate in their niche (28). In homeostatic condition, HSC can also exit from BM to blood and return to other functional niches in BM via blood flow (28). The defects of HSC/HPC trafficking in homeostasis and of HSC/HPC homing to the BM after transplantation, which we observed when Fancg is deleted, illustrate the crucial role of Fancg in HSC physiology. Interestingly, a recent paper has similarly reported a defective homing of human hematopoietic progenitors in cells from patients of FA group A (46).

The chemokine CXCL12 plays a crucial role in homing to the BM and in migration through cell adhesion and chemotraction (26). Conditional invalidation of its receptor CXCR4 results in the decrease in the pool of HSC, the exit of quiescence and the decrease in the repopulating potential of HSC (34,47). The cyclin-dependent kinase inhibitor p57^kip2, identified as a potential regulator of HSC quiescence (48–51), is one of the targets downstream of CXCR4 signaling pathway (47). Interestingly, we observed a lack of response to CXCL12-CXCR4 signaling in Fancg−/− LSK, and also a decrease in the expression of p57^kip2 and an alteration of the quiescence. Collectively, these data suggest the potential involvement of the CXCL12-CXCR4 pathway in FA deficiency, although another group previously reported in lineage-negative hematopoietic progenitors no statistically difference comparing CXCL12-mediated migration of Fancg−/− progenitors with WT progenitors (52). Moreover, the hyperproliferative Cxcr4−/− HSC sustained hematopoiesis throughout adult life and mice were not aplastic, but showed impaired engraftment after transplantation due to an incapacity of CXCR4-deficient HSC to home to the BM niche (47). Those traits of Cxcr4−/− mice are reminiscent of the phenotype of the mice deficient for the FA genes. These evidences suggest that an impairment of the CXCR4 signaling pathway could play a role in the deficiency of Fancg−/− HSC to repopulate BM and that FA proteins could interact with downstream effectors of this pathway.

The data presented here show an impairment of the functionality of HSC/HPC in Fancg−/− mice. The expression of several genes involved in the quiescence and the self-renewal of HSC were dysregulated, and our data suggest that FA deficiency could alter downstream effectors of the CXCR4/CXCL12 signaling involved in migration and homing. In addition to the role of the FA pathway in DNA repair, this work highlights that HSC defects in FA pathology could also result from the alteration of fundamental functions of HSC physiology.
MATERIALS AND METHODS

Mice

Fancg−/− mice were generated in a 129Ola/FVB background (4). Fancg+/− heterozygotes were backcrossed six times with C57BL/6 and EGFP transgenic mice (53) (C57BL/6-TgN(beta-act-EGFP)01Os—generous gift of Dr Okabe) in order to generate heterozygous Fancg+/− EGFP mice. Then they were intercrossed to generate homozygous Fancg−/− EGFP mice. All data presented in this study were obtained with offspring from heterozygous parents of the sixth C57BL/6 backcross. Unless specified otherwise, 2–4-month-old mice were used for experimentation. In order to examine the effect of dietary supplementation with the antioxidant NAC, NAC (1 mg/ml) was added to drinking water of Fancg-deficient mice and WT mice from fertilization continuously throughout the life. All animal procedures reported in this paper were carried out in accordance with French Government regulations (Services Vétérinaires de la Santé et de la Production Animale, Ministère de l’Agriculture).

Flow cytometry

Femurs and tibias were flushed. After red blood cells lysis, BM mononuclear cells were phenotyped using antibodies from Becton Dickinson unless otherwise indicated. The analysis of HSC/HSPs subsets was performed using biotin-conjugated anti-mouse lineage antibodies (Miltenyi Biotec), PC7-conjugated streptavidin, fluorescein isothiocyanate (FITC)-conjugated anti-Sca-1, allophycocyanin (APC)-conjugated anti-c-Kit, phycoerythrin (PE)-conjugated Flk-2 and Pacific-blue conjugated anti-CD45 antibody (BD Pharmingen) and PE-conjugated anti-Sca-1, allophycocyanin (APC)-conjugated anti-c-Kit, phycoerythrin (PE)-conjugated Flk-2 and Pacific-blue conjugated anti-CD45 antibody (BD Pharmingen), PC7-conjugated APC-conjugated anti-c-Kit and FITC-conjugated anti-CD34. For CLP analysis, cells were stained with biotin-conjugated anti-mouse lineage antibodies (Miltenyi Biotec) together with Texas-Red-conjugated streptavidin, and then incubated with PE-conjugated anti-Sca-1, PC7-conjugated anti-Fcγ, APC-conjugated anti-c-Kit and FITC-conjugated anti-CD34. To study lymphoid and myeloid lineage, BM cells were stained directly with conjugated anti-CD4-PC5, anti-Gr-1-APC or anti-Mac-1-APC antibodies. For LSK cell sorting, BM mononuclear cells were depleted of lineage-committed cells using the MACS and cell separation columns (Miltenyi Biotec). Lin− cells were stained with anti-Sca-1 FITC and anti-c-Kit APC antibodies, fixed in paraformaldehyde 1%, permeabilized with 0.1% Triton X-100 and stained with DAPI and anti-Ki-67 PE, (BD Pharmingen). Analysis was performed using a LSRII flow cytometer.

Migration experiments

Transwell chambers (Costar) were used to assess cell migration through filters (pore size 6 μm). 104 sorted LSK cells were plated with RPMI media (GIBCO) onto polycarbonate filter. CXCL12 was added to the bottom chamber at 100 ng/ml. After 4 h, cells in the bottom side chamber were stained with anti-CD45 antibody (BD Pharmingen) and LSK cells were counted by flow cytometry using the TruCount™ (BD Biosciences) methodology.

Homing assay

Flow-sorted EGFP-positive LSK cells from Fancg−/− or WT control mice were transplanted (1000 cells/mouse) by retro-orbital route into lethally irradiated (10 Gy) WT recipients that were irradiated 24 h before transplantation. Eighteen hours after injection, the EGFP-positive LSK cells that homed to the BM were detected and counted by cellular imaging using a fiber-optic imaging system as recently described (27). Briefly, the knee of anesthetized transplanted mice was flexed and superficially pierced with a 23-gauge needle through the joint. Through this route, the 45-degree beveled S300 flexible microprobe (Mauna Kea Technologies) was entered and moved slowly inside the femoral cavity all the way up to the femoral head. This microprobe containing 10 000 optical fibers that carry light from a continuous laser source at 488 nm to the living tissue. Fluorescence emitted by excitation of the GFP protein is detected and images are reconstructed in real time (rate of acquisition 12 frames/s). The spatial resolution allows the detection of single cells (8–10 μm). Video data acquisitions and analyses were performed using the Diva imaging system (Mauna Kea Technologies). Video data acquisitions were performed using the Fiber-optic imaging system (Mauna Kea Technologies). Video data acquisitions and analyses were performed using the Diva imaging system (Mauna Kea Technologies).

In vivo competitive reconstitution assay and in vitro progenitor assay

For competitive reconstitution assay, 250 000 BM cells from either EGFP Fancg−/− or EGFP WT were mixed with 250 000 WT BM cells. The suspensions were transplanted by retro-orbital or intra-femoral route into lethally irradiated (10 Gy) WT recipients. Analysis by flow cytometry of peripheral blood was performed 4, 8, 12 and 16 weeks after transplantation according to CD45 (anti-CD45 antibody-BD Pharmingen) and EGFP fluorescence donor-derived markers in order to examine hematopoietic regeneration. To study lymphoid and myeloid chimerism, cells were stained directly with conjugated anti-CD4-PC5, anti-CD8-PC5, anti-Gr-B220-PC5, anti-Gr-1-APC or anti-Mac-1-APC antibodies. Competitive stem cell reconstitution assay was also performed with 2000 LSK cells from either EGFP Fancg−/− or EGFP WT mice mixed with 250 000 BM cells of WT mice. For in vitro progenitor assay, methylcellulose cultures were performed from 50 000 and 25 000 BM cells or 150 and 300 sorted LSK cells using MethoCult® M3334 and M3434 according to the manufacturer’s protocol (Stem Cell Inc).
performed with the CellVizio 488 and Image Cell software (Mauna Kea Technologies).

**In vitro wounding assay using live video microscopy**

Primary MEF were obtained using WT and *Fancg*−/− embryos at day 13.5 of gestation. MEF were plated and frozen in liquid nitrogen as passage 0 (P0). For scratch wound closure motility assays, MEF as passage 1 (P1) obtained from four different WT and four different *Fancg*−/− embryos were used. *Fancg*−/− and WT MEF were seeded in plates and then wounded by scratching with pipette tip. Live microscopy was carried out using an inverted microscope (Olympus IX81) placed in an incubator chamber maintained at 37°C, and coupled with a coolscan HQ camera (Princeton Instruments) controlled by Metamorph software. Levels of CO₂ (5%), O₂ (19%) and humidity (95% relative humidity) were controlled during all the experiments by an active gas supply system (The Brick; LIS). For phase-contrast time lapse video microscopy, images were taken using a 10× objective. All images were converted in 8-bit files before being assembled with Metamorph software. To quantify cells in S-phase, MEF (MEF isolated from eight different embryos were used, n = 4/genotype) were pulse labeled with BrdU for 90 min and BrdU proliferation assay was performed by flow cytometry using the BrdU-APC flow kit (BD Pharmingen).

**Apoptosis assay and ROS production**

BM mononuclear cells were stained using biotin linkage antibody cocktail (Milenyi Biotec), streptavidin-PC7, Sca-1-FITC and c-Kit-APC antibodies (BD Pharmingen). Apoptosis was analyzed using a LSRII by staining LSK cells with annexin V-PE and 7-AAD (BD Pharmingen). To assess apoptosis in EGFP-positive LSK cells after intrafemoral transplantation, BM mononuclear cells from recipient mice were stained with biotin-conjugated anti-mouse lineage antibodies (Miltenyi Biotec), permeabilized (0.2% Triton X-100), blocked (2% BSA and 2% fetal bovine serum) and stained with anti-phosphorylated Ser139 H2AX antibodies (Millipore), Alexa488- or Alexa594-conjugated secondary antibodies (Invitrogen) and counterstained with DAPI (Sigma). Images were captured using a Nikon AIR confocal laser scanning microscope system attached to an inverted ECLIPSE Ti (Nikon Corp.) and a 60× objective (NA 1.4). DAPI, Alexa488 and Alexa594 fluorochrome were, respectively, excited with 405, 488 and 561 nm lasers and fluorescence was collected using 450/50, 525/50 nm and 595/50 filter sets. Image analysis was conducted using NIS-Elements software (Nikon Corp.). LSK cells from four WT and four *Fancg*−/− mice were sorted and analysed. γ-H2AX foci from at least 50 nuclei per animal were measured.

**RT-PCR**

mRNA were prepared from 10⁴ flow-sorted LSK cells using microMACS mRNA isolation kit (Miltenyi Biotec), and reverse transcribed using Quantitect enzyme (Qiagen). The cDNA was subjected to real-time PCR using Power SYBR Green Master Mix and Taqman Gene Expression Master Mix (Applied Biosystem) in 7000 Sequence Detection System. Amplification of GAPDH was used for sample normalization. Primer sequences are as follows: CXCR4 (5′-ATGGAACCGATCAGTGAG-3′, lower 5′-TGACAGAAAAACACCTTCC-3′, p18 (5′-GGACCTTAGAGCAATCTACT-3′, lower 5′-GGGAAACCCTGCTGGAAAT-3′), c-myc (5′-TTCCGATTCTCTTTGGCCTT-3′, lower 5′-AATTAGGGCTGTACCGGTCG-3′), p21 (5′-TCCGACACTTCAACCCACA-3′, lower 5′-CAGAGAGACACCGCCACAC-3′), Hif-1α (5′-CCCCATTCCTCATCGCCAACA-3′, lower 5′-CGGCTCTATAACCCACTCAACT-3′), Mef (5′-GAGCCCTTGGATGAGAAACA-3′, lower 5′-GTCGCTTCCCATCCCCATTTA-3′), Bmi-1 (5′-ATGGGTTCGCCAGGTTCTTGTT-3′, lower 5′-GTAGTGGGGTCCTCGCAGTT-3′), N-Cad (5′-AGCCGCAATGACTGTGACCTT-3′, lower 5′-GCCCTGGTTCCTGGGA-3′), EGR1 (5′-AGCCGACGGCAACAACCTCCTA-3′, lower 5′-TATCTGTTGGGCTGGCAAGT-3′), p53 (5′-AGGAGCAGGGAGCAGAATC-3′, lower 5′-CAGACCTGAATTTGGAGGTCG-3′), Rac1 (5′-ATGACATCCCACCCTCTTT-3′, lower 5′-CCACTTGCAGGAGATTT-3′), TGFβ (5′-AGTATGACGAACACCCACC-3′, lower 5′-GCCGTCGACCAATCTTTA-3′), p27 (5′-ACCTCCAGGAGACCA-3′, lower 5′-GCCCTGGTTCCTGGGA-3′), p19 (5′-GAGCCCTTGGATGAGAAACA-3′, lower 5′-GTCGCTTCCCATCCCCATTTA-3′), Hif-1α (5′-CCCCATTCCTCATCGCCAACA-3′, lower 5′-CGGCTCTATAACCCACTCAACT-3′), Mef (5′-GAGCCCTTGGATGAGAAACA-3′, lower 5′-GTCGCTTCCCATCCCCATTTA-3′).

**Immunofluorescence**

Sorted LSK cells were deposited on slides, fixed (1% paraformaldehyde), permeabilized (0.2% Triton X-100), blocked (2% BSA and 2% fetal bovine serum) and stained with anti-phosphorylated Ser139 H2AX antibodies (Millipore), Alexa594- or Alexa488-conjugated secondary antibodies (Invitrogen) and counterstained with DAPI (Sigma). Images were captured using a Nikon AIR confocal laser scanning microscope system attached to an inverted ECLIPSE Ti (Nikon Corp.) and a 60× objective (NA 1.4). DAPI, Alexa488 and Alexa594 fluorochrome were, respectively, excited with 405, 488 and 561 nm lasers and fluorescence was collected using 450/50, 525/50 nm and 595/50 filter sets. Image analysis was conducted using NIS-Elements software (Nikon Corp.). LSK cells from four WT and four *Fancg*−/− mice were sorted and analysed. γ-H2AX foci from at least 50 nuclei per animal were measured.

**Statistical analysis**

Data are mean ± SEM. Unpaired Student’s t-test (one tail) was used to determine statistical significance.

**SUPPLEMENTARY MATERIAL**

Supplementary Material is available at *Human Molecular Genetics* online.
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Conflicts of Interest statement. None declared.

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