Bone marrow niches of germline FANCC/FANCG deficient mice enable efficient and durable engraftment of hematopoietic stem cells after transplantation

Efficient and durable hematopoietic stem cell (HSC) engraftment is essential for successful HSC transplantation (HSCT). Multiple previous studies had demonstrated active participation of specialized bone marrow (BM) niches in regulating HSC engraftment during HSCT.[1-3] Recently, studies using models of human inherited BM failure syndromes (iBMFS) have demonstrated that BM niche dysfunction contributes to BM failure (BMF) pathogenesis and HSC loss.[4-7] However, whether impaired niche cell survival or function diminishes healthy donor HSC engraftment following curative HSCT for these iBMFS remains undefined. Fanconi anemia (FA) is a BMF associated with germline mutations in one of 23 known FA genes that contribute to intrastrand DNA crosslinks and homologous recombination.[8,9] Several mouse strains with germline FA gene deletion have been established during the last decade.[10] Specifically, *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ double knockout mice have been described as developing BMF spontaneously.[11] Reduction in HSC has now also been described in mice lacking FANCG alone.[12]

A recent study in this journal explored whether functional defects in mesenchymal stem/progenitor cells (MSPC) from BM niches of *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ mice contribute to BMF development.[5] However, it is still not clear whether germline deficiency of FA genes within MSPC impairs the capacity of recipient BM niches to engraft functionally normal donor HSC post transplantation. Here we demonstrate that BM niches of mice with germline deficiency of FANCC and/or FANCG remain capable of efficient and durable engraftment of donor HSC post HSCT.

Given that BMF arises in FA following exposure to environmental stress,[13] we first analyzed whether *Fancc*$^{-/-}$, *Fancg*$^{-/-}$, and *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ mice of ages suitable for HSCT developed BMF. None of these FA models demonstrated deficiency in peripheral blood leukocyte, neutrophil, hemoglobin or platelet counts compared with wild-type (WT) mice up to 40 weeks of age (Figure 1A). BM cellularity was also not reduced in *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ *versus* WT mice up to 40 weeks of age (Figure 1B and C). Quantification of HSC/progenitor subsets showed no changes in percentages of LSK (Lin$^-$Sca-1$^+$c-Kit$^+$), long-term HSC (LT-HSC), short-term HSC (ST-HSC), multipotent progenitor (MPP) and common myeloid progenitor in FA mice compared to WT (Figure 1D), although *Fancg*$^{-/-}$ and *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ mice exhibited lower percentages of common lymphoid progenitors. Consistent with previous studies,[5,11] HSC/progenitors from *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ mice exhibited decreased clonogenic potential *versus* WT in colony-forming unit (CFU) assays (Figure 1E). In apop-

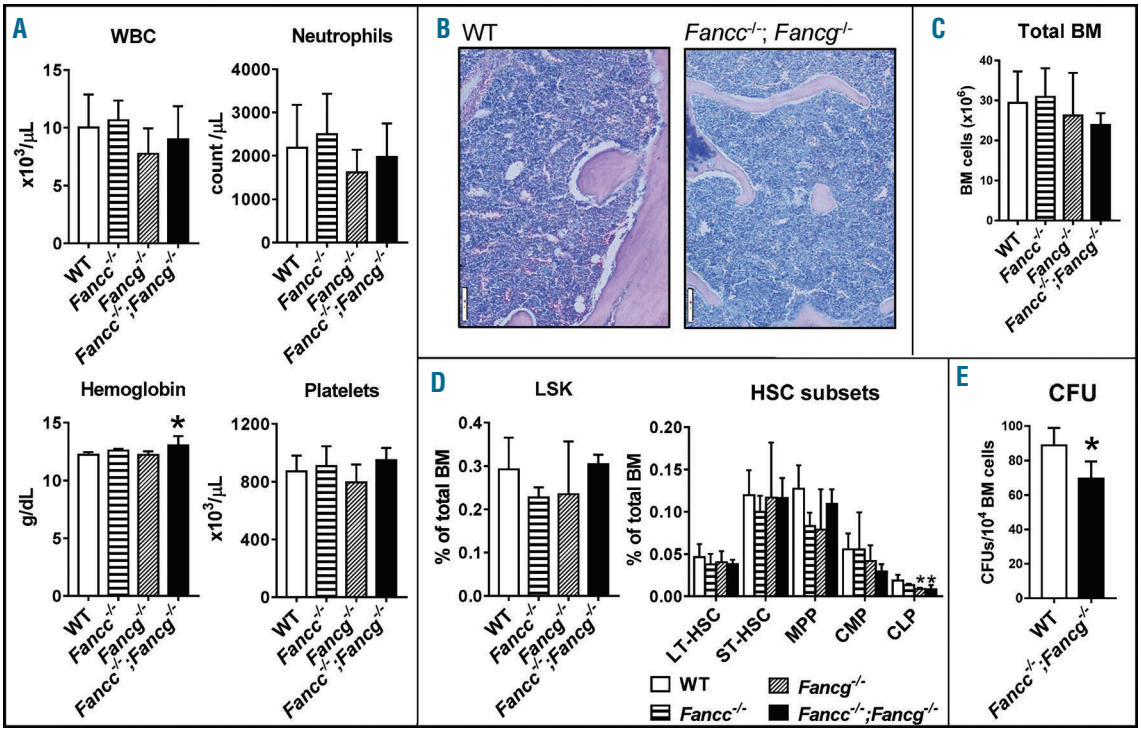

Figure 1. Mice with germline deficiency of Fancc and/or Fancg display normal peripheral blood counts, bone marrow (BM) cellularity and hematopoietic stem/progenitor cell frequencies. (A) Complete blood count (CBC) analysis of white blood cells (WBC), neutrophils, hemoglobin and platelets in WT, *Fancc*$^{-/-}$, *Fancg*$^{-/-}$ and *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ mice. Mean±Standard Deviation (SD). N=4-8 mice per group. (B) Hematoxylin & Eosin staining of BM section from a 6-month old wild-type (WT) and *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ double mutant mouse. (C) Total number of BM cells in 6-10-month old WT, *Fancc*$^{-/-}$, *Fancg*$^{-/-}$ and *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ mice (mean±SD). N=4 mice per group. (D) Frequencies of LSK (Lin$^-$Sca-1$^+$c-Kit$^+$), LT-HSC (Lin$^-$Sca-1$^+$c-Kit$^+$CD48$^-$CD150$^+$), ST-HSC(c-Kit$^+$Lin$^-$Sca-1$^+$CD135$^-$ CD34$^+$), multipotent progenitor (MPP, Lin$^-$Sca-1$^+$c-Kit$^+$CD135$^+$), common myeloid progenitor (CMP, Lin$^-$Sca-1-c-Kit$^+$CD34$^+$) and common lymphoid progenitor (CLP, Lin$^-$Sca-1$^+$c-Kit$^+$CD127$^+$) in 6-10-month old WT, *Fancc*$^{-/-}$, *Fancg*$^{-/-}$ and *Fancc*$^{-/-}$*; Fancg*$^{-/-}$ mice, expressed as the percentage of total BM cells (mean±SD). N=3-4 mice per group). (E) Total numbers of colony-forming units (CFU) per 10^4 BM cells from WT and *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ mice (mean±SD). N=4 mice per group. *P<0.05 compared with WT.

tosis and cell cycling assays, we found that, compared to WT, *Fancc*$^{-/-}$ *and Fancc*$^{-/-}$*/Fancg*$^{-/-}$ mice possessed fewer CD48^{-} LSK exhibiting early apoptosis (Annexin-V^{+}7AAD^{+}) and an increased percentage of CD48^{-} LSK from *Fancg*$^{-/-}$ mice were in S/G2/M phase (*Online Supplementary Figure S1B and C*). A recent study demonstrated that polyinosinic-polycytidylic acid (pIpC) administration, which mimics viral infection through generating type I interferon responses, can induce BMF in Fanca$^{-/-}$ mice.[13] We injected pIpC into *Fancc*$^{-/-}$, *Fancg*$^{-/-}$, and *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ mice. Compared with untreated or pIpC-treated WT, none of the pIpC-treated FA models devel-

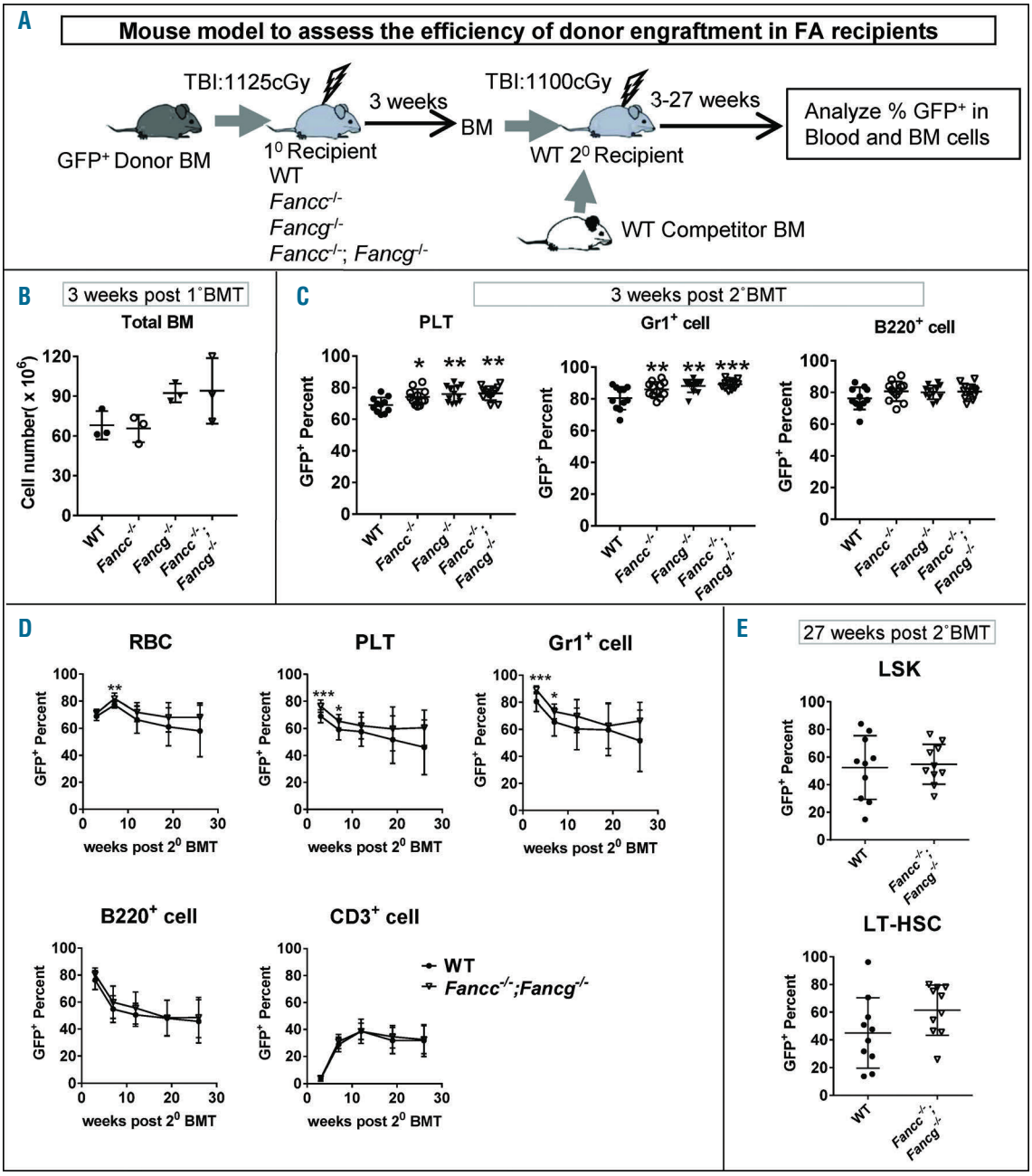

Figure 2. ***Fancc*$^{-/-}$*;Fangc*$^{-/-}$ **double mutant mice display no reduction in the efficiency of long-term (LT)-hematopoietic stem cell (HSC) engraftment after HSC transplantation (HSCT) compared to wild-type (WT) controls, but show enhanced early hematopoietic progenitor engraftment.** (A) Primary Fanconi anemia (FA) or WT recipients were transplanted with 5x10^{6} whole bone marrow (BM) cells from H2K-GFP donor mice after receiving 1125cGy total body irradiation (TBI). The efficiency of GFP^{+} donor engraftment was assessed at three weeks after 1ry BM transplantation (BMT) using competitive secondary transplantation assay, in which equal volumes (20% of cumulative BM volume from bilateral hindleg bones) of whole BM from FA or WT 1ry recipients was transplanted with 10^{5} whole BM cells from unirradiated WT competitor mice into irradiated WT 2nd recipients. (B) Total BM cell number of *Fancc*$^{-/-}$, *Fangc*$^{-/-}$, *Fancc*$^{-/-}$*;Fangc*$^{-/-}$ and WT 1ry recipients at three weeks after 1ry BMT. (C) Donor engraftment of early hematopoietic progenitors in WT and FA 1ry recipients examined by GFP^{+} cell percentage in platelets (PLT), Gr1 myeloid cells and B220^{+} B cells in 2ry recipient mice at three weeks post 2ry BMT. (D) Assessment of donor HSC engraftment in WT and FA primary recipients by GFP^{+} cell percentage in multiple mature blood lineage including red blood cells (RBC), PLT, Gr1^{+} myeloid cells, B220^{+} B cells and CD3^{+} T cells in 2ry recipient mice up to 27 weeks after 2ry BMT. (E) GFP^{+} reconstitution in the populations of LSK (Lin^{-}Sca-1^{+}c-Kit^{+}) and LT-HSC(Lin^{-}Sca-1^{+}c-Kit^{+}CD48^{-}CD150^{+}) in the BM of 2nd recipient mice at 27 weeks after 2nd BMT. **P*<0.05; ***P*<0.01; ****P*<0.001 compared with WT.

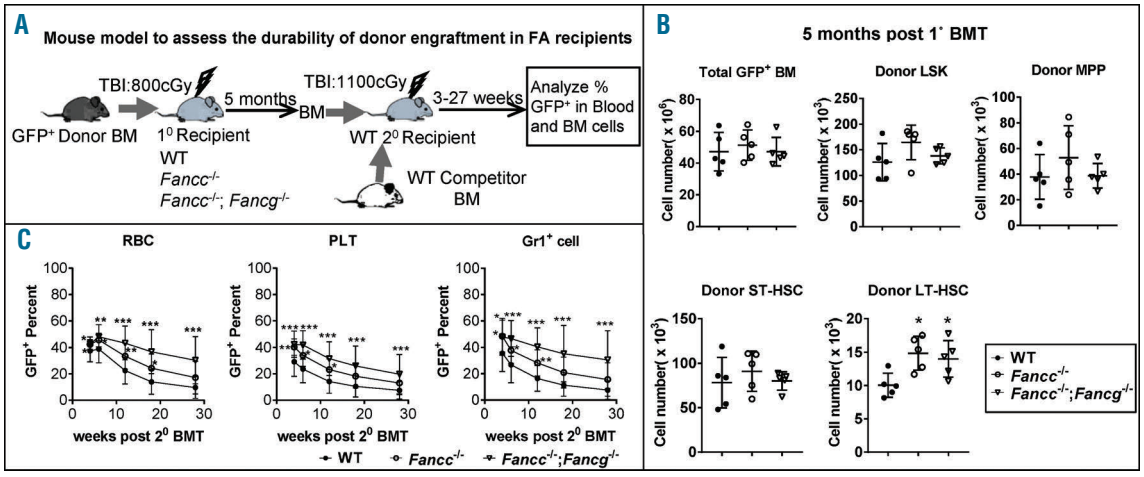

Figure 3. Mice with germline deficiency of FANCC and/or FANCG have durable hematopoietic stem cell (HSC) engraftment after transplantation. (A) Primary wild-type (WT) and Fanconi anemia (FA) recipients were transplanted with 5x10^6 whole bone marrow (BM) cells from H2K-GFP donor mice after receiving 800 cGy total body irradiation (TBI). Durability of GFP$^+$ donor engraftment was assessed at five months after 1ry BM transplantation (BMT) by competitive secondary transplantation assay, in which 10^6 from each FA or WT 1ry recipients were transplanted with 5x10^5 whole BM cells from unirradiated WT mice into irradiated (1100cGy) WT 2ry recipients. (B) The BM of 1ry mice recipients were analyzed at five months after 1st BMT. Scatterplots represent the cell cumber quantitation of total GFP$^+$ BM, as well as GFP$^+$ donor LSK (Lin$^-$Sca-1$^+$c-Kit$^+$), Lin$^-$Sca-1$^+$c-Kit$^+$CD135$^+$ MPP, c-Kit$^+$Lin$^-$Sca-1$^+$ CD135$^-$CD34$^+$ ST-HSC and Lin$^-$Sca-1$^+$ c-Kit$^+$CD150$^+$CD48$^-$ LT-HSC in WT, *Fancc*$^{-/-}$ and *Fancc*$^{-/-}$;*Fancg*$^{-/-}$ recipients. *P<0.05 compared to WT. (C) Donor engraftment in primary WT, *Fancc*$^{-/-}$ and *Fancc*$^{-/-}$;*Fancg*$^{-/-}$ recipients was assessed by GFP$^+$ cell percentage in blood lineages including red blood cells (RBC), platelets (PLT) and Gr1$^+$ myeloid cells in 2ry recipient mice at 3-27 weeks after 2ry BMT. *P<0.05; **P<0.01; ***P<0.001 compared with WT.

oped deficits in blood counts or BM cellularity (*Online Supplementary Figure S1D and E*).

Given the lack of appreciable BMF in *Fancc*$^{-/-}$, *Fancg*$^{-/-}$ and *Fancc*$^{-/-}$;*Fancg*$^{-/-}$ mice within the age ranges at which these mice would be used as HSCT recipients, we reasoned that we could use these models to test the impacts of FANCC and/or FANCG deficiency directly within mesenchymal niche cells on the capacity of the BM niche to facilitate efficient post-transplantation HSC engraftment, without the confounding influence of BMF on recipient niche cell function during HSCT. We thus transplanted GFP$^+$ WT whole BM into WT and *Fancc*$^{-/-}$;*Fancg*$^{-/-}$ recipients two days after 1125 cGy total body irradiation (TBI). At three weeks post HSCT, we assessed GFP$^+$ BM reconstitution, finding that, rather than having impaired BM recovery following HSCT, *Fancg*$^{-/-}$ and *Fancc*$^{-/-}$;*Fancg*$^{-/-}$ recipients exhibited a trend towards more rapid BM cell recovery (P=0.08 and P=0.14, respectively) (Figure 2B). We then conducted competitive secondary (2ry) transplantation assays, in which the transplanted GFP$^+$ BM harvested from WT or FA recipients three weeks post-initial (1ry) HSCT were transplanted with 10^5 BM cells into irradiated secondary WT recipients (Figure 2A). At three weeks post-2ry BMT, GFP$^+$ reconstitution of myeloid (GR1$^+$) and platelet lineages, but not B lymphocytes (B220$^+$), were increased in 2ry recipients of BM derived from FA *versus* WT primary recipients (Figure 2C), indicating that FA niches engrafted committed myeloid hematopoietic progenitor cells more efficiently than WT niches. To understand why FA niche leads to more rapid progenitor engraftment, we evaluated the expression of CXCL12, the chemokine critical in attracting HSC into BM niches. We found that residual BM stromal cells from irradiated *Fancg*$^{-/-}$ *versus* WT mice exhibited higher CXCL12 transcript expression (*Online Supplementary Figure S2B*). From 12-27 weeks post-2ry BMT, time points when hematopoiesis is driven specifically by donor LT-HSC derived from the WT competitor or primary recipient BM, secondary recipients receiving WT and FA primary recipient BM showed similar GFP$^+$ reconstitution of blood cell lineages and BM LSK cells (Figure 2D and E, *Online Supplementary Figure S2A* and *data not shown*), indicating that FANCC and/or FANCG deficiency within HSCT recipient niche caused no deficits in donor HSC engraftment efficiency within the first three weeks post HSCT, even after myeloablative TBI. In fact, 2ry recipients receiving *Fancc*$^{-/-}$;*Fancg*$^{-/-}$ 1ry recipient BM showed a trend toward higher GFP$^+$ reconstitution in GR1$^+$ cells in blood (P=0.08) and LT-HSC in BM (P=0.11) than those receiving WT 1ry recipient BM (Figure 2D and E), which may reflect myeloid-bias propagated by FA niche. Taken together, compared to WT, BM niches in *Fancc*$^{-/-}$, *Fancg*$^{-/-}$ and *Fancc*$^{-/-}$;*Fancg*$^{-/-}$ mice demonstrate more rapid BM reconstitution, enhanced committed progenitor engraftment, and no deficits in initial LT-HSC engraftment efficiency during the first three weeks post HSCT.

Next, we asked whether FANCC and/or FANCG deficiency in the BM niche impacted durable WT HSC engraftment post-HSCT. FA and WT 1ry recipients received GFP$^+$ WT BM following a reduced-dose of 800cGy-TBI due to concerns regarding long-term non-hematologic toxicity in the FA models. At five months post-1ry BMT, 1ry recipient BM was analyzed for immunophenotypic HSC/progenitors (Figure 3A). We found no reductions in total donor BM cell and any HSC/progenitor subsets in FA recipients *versus* WT recipients. In fact, all FA recipients displayed higher donor LT-HSC numbers than WT recipients, and *Fancg*$^{-/-}$ recipients also engrafted more GFP$^+$ LSK and ST-HSC (Figure 3B and *Online Supplementary Figure S3B-D*). Functional HSC in 1ry recipients was further analyzed by an additional competitive secondary transplantation assay. First recipient BM (1x10^6) together with 5x10^5 whole BM from unirradiated WT competitor mice was transplanted into irradiat-

ed (1100 cGy) WT 2ry recipients (Figure 3A). Secondary recipients of FA *versus* WT 1ry recipient BM displayed no deficiency in GFP$^+$ reconstitution in multiple blood lineages between 3-27 weeks post-2ry BMT (Figure 3C and *Online Supplementary Figure S3E*). In fact, GFP$^+$ reconstitution of multilineage blood cells in 2ry recipients of *Fancc*$^{-/-}$ and *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ recipient BM was enhanced compared to those of WT recipient BM, though this enhancement may be caused by more residual host cells and less than complete (80%) GFP$^+$ reconstitution in WT 1ry recipients BM compared with FA 1st recipients BM (*Online Supplementary Figure S3A*), due to the TBI dose used (800 cGy). Regardless, taken together, these data demonstrate that germline deficiency of FANCC and/or FANCG did not impair the durability of donor engraftment post HSCT.

We have demonstrated that germline deficiency of FANCC and/or FANCG did not impair the capacity of the BM niche to promote efficient and durable HSC engraftment post transplantation. *Fancc*$^{-/-}$, *Fancg*$^{-/-}$ and *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ mice demonstrated normal blood counts up to 40 weeks, in line with a previous study of *Fancg*$^{-/-}$ mice.[12] We also found no deficits in BM cellularity or immunophenotypic HSC/progenitors in mice with FANCC and/or FANCG deficiency even with pIpC-induced HSC cycling. However, *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ HSC/progenitors showed reduced clonogenic potential consistent with previous reports.[5,11] Comparing efficiency and durability of HSC engraftment in WT *versus* FA HSCT recipients by assessing functional HSC content at three weeks and five months post HSCT through competitive secondary transplantation, we found no deficiency in HSC engraftment in FA recipients. Previously, Zhou *et al*. suggested that *Fancc*$^{-/-}$*;Fancg*$^{-/-}$ recipients have decreased niche-mediated donor HSC engraftment, based on their observation of decreased CFU number in BM after transplantation with WT BM.[5] It is possible that this decreased CFU number was relative, and could be explained by the more rapid BM reconstitution and increased hematopoietic progenitor engraftment seen in FA recipients initially following HSCT, which could lead to true colony-forming HSC representing a lower percentage of BM cells at the time studied. The higher CXCL12 expression we identified in the irradiated *Fancg*$^{-/-}$ BM environment may explain why FA recipients have increased hematopoietic progenitor engraftment/expansion initially after HSCT, though further comparisons of molecular and cellular profiles within FA *versus* WT recipient BM niches are needed.

While survival rates of HSCT in FA have improved over the last two decades, strategies to further reduce conditioning intensity could reduce complications and improve quality of life.[14] BM niche dysfunction, either due to pre-existing BMF or the direct impact of FA gene deficiency on niche cells poses a theoretical obstacle to stable engraftment with further reduced conditioning intensity for HSCT in FA. Our study has demonstrated for the first time that the functional capacity of recipient BM niche to stably engraft donor HSC remains intact despite FANCC and/or FANCG deficiency within the niche. To determine whether BMF itself impacts niche function during HSCT for FA, new FA models in which severe BMF can reproducibly be induced are required. One such approach could be to generate humanized BM niche xenografts by transplanting FA patient-derived MSPC and HSC/progenitors into immunodeficient mice, as previously described.[15]

Ji Zha,[1] Lori Kunselman,[1] Jian-Meng Fan,[1] and Timothy S. Olson[1,2,3]*

[1]Comprehensive Bone Marrow Failure Center, Children's Hospital of Philadelphia, Philadelphia; [2]Blood and Marrow Transplant Section, Division of Oncology, Department of Pediatrics, Children's Hospital of Philadelphia, Philadelphia and [3]Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA, USA

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Correspondence: TIMOTHY S OLSON.
olsont@email.chop.edu.
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